PhD THESIS

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REGULATION OF CUCUMBER MOSAIC VIRUS MOVEMENT PROTEIN LOCALIZATION AND ITS ROLE IN VIRUS SYMPTOM DEVELOPMENT

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1. INTRODUCTION AND OBJECTIVES

Studying plant viruses provides us with more and more information about the molecular background of virus-host plant interactions. This includes understanding how viruses can enter plant tissues, how they replicate there, and how the host plant responds to the infection. To facilitate successful infection, the majority of viruses encode a specific protein for this purpose, known as the movement protein (MP). The main task of movement proteins is to assist the viral genome in moving from the initially infected cell to neighboring, still healthy cells, and eventually, to transport it through the phloem to all parts of the plant. Just like the viruses themselves, these movement proteins can also vary and employ diverse strategies to carry out their main function. By understanding how movement proteins operate, we can better understand the development of plant virus infections and identify the key factors in virus spread. This, in turn, may contribute to the prevention of viral diseases and the development of defense strategies in the future.

The objectives of this study are as follows:

1. Identification of potentially phosphorylatable amino acids IN CMV movement protein (MP) using *in silico* methods.
2. Examination of expressed CMV MP phosphorylation using mutant viruses containing targeted point mutations.
3. Generating CMV mutants using infectious CMV clones, which encode alanine or asparagine at positions 28^{th} and 120^{th}, thus modeling the phosphorylated and non-phosphorylated states of the MP. Inoculation of tobacco (*Nicotiana benthamiana* Domin) test plants with mutant infectious clones and the determination of the virulence of the mutant viruses. After the verification of the stability of the mutations, we aimed to purify the mutant virions for further investigations.

4. Observation of the difference between symptom development of the wild type and mutant CMV on tobacco (*Nicotiana tabacum* L. cv. Xanthi-nc), *Chenopodium murale* L., and cucumber (*Cucumis sativus* L. cv. Szenzáció). Analysis of the observed differences using various molecular virology methods.
5. Analysis of the effect of MP phosphorylation on subcellular localization using *Agrobacterium*-mediated transient gene expression and confocal laser-scanning microscopy.

2. MATERIALS AND METHODS

2.1 Experimental materials

2.1.1. Virus strains

The Rs-CMV isolate used in our experiments was isolated by dr. Pál Salamon from *Raphanus sativus* L. The infectious clones were generated by Divéki et al. (2004) (pRs1, pRs2, pRs3).

2.1.2. Plant material

For the experiments, we used test plants grown under greenhouse conditions as listed below: *N. benthamiana* Domin, *N. tabacum* L. cv. Xanthi-nc, *Chenopodium murale*, *C. sativus* L. cv. Szenzáció. The *Nicotiana* and *C. murale* plants were grown in growth chambers under the following temperature and light conditions: 16 h light 23 ºC, 8 h dark 20 ºC. The cucumber plants were grown under the same light conditions, but with temperatures of 26 ºC and 23 ºC.

2.1.3. Bacterium strains

For the experiments, we used *E. coli* strains DH5α, TG90 and BL21(DE3). For the agroinfiltration experiments, we used *A. tumefaciens* strain C58C3.

2.1.4. Plasmids

For cloning we used pGEM® T-Easy (Promega) vector. For the protein expression experiments we used pET28a (Sigma-Aldrich) vector, and for the agroinfiltration experiments we used pBin61 binary vector (SILHAVY és mtsai., 2002).

4.2. Methods

4.2.1. *In silico* phosphorylation prediction

We used a free online phosphorylation site prediction software, NetPhos 3.1, for phosphorylation site prediction. (https://services.healthtech.dtu.dk/service.php?NetPhos-3.1). The program examines the protein based on various protein databases and recognizes 17 kinase recognition sites. Using this method, the program determines potentially phosphorylatable amino acids (Ser, Tyr, Thr). For these amino acids, the program assigns a score between 0-1 at the end of the analysis (prediction score), indicating the likelihood of phosphorylation. We used the default settings of the program, where phosphorylation of the amino acid can be considered valid if the prediction score is above 0.5.

4.2.2. Creating movement protein mutants containing alanine and aspartic acid

4.2.2.1. Creating mutant infectious clones

The mutant infectious clones containing alanine and aspartic acid (MP/S28A, MP/S28D, MP/S120A, MP/S120D) were created with PCR based mutagenesis using pRs3 infectious clone. The used primer pairs are presented in Table 1. A two-step PCR method was used when creating the mutant infectious clones. For clones MP/S28A and MP/S28D, the 5' end of pRs3 was amplified using primers 57 and 420 (PCR conditions: 95 ºC 5 min, 30 cycle 95 ºC 30 sec, 55 ºC 30 sec, 72 ºC 1 min, and 72 ºC 10 min). The longer 3' end region containing the single nucleotide point mutations were amplified with primers 418 and 43 in the case of MP/S28A, and primers 419 and 43 in the case of MP/S28D (PCR conditions: 95 ºC 5 min, 30 cycle 95 ºC 30 sec, 55 ºC 30 sec, 72 ºC 6 min, and 72 ºC 10 min). The two amplified regions were connected with overlap PCR using primers 57 and 43 (PCR conditions: 95 ºC 5 min, 30 cycle 95 ºC 30 sec, 60 ºC 30 sec, 72 ºC 1 min, and 72 ºC 10 min).

For clones MP/S120A and MP/S120D the 5' end of pRs3 was amplified using primer pair 57 and 423 (PCR conditions: 95 ºC 5 min, 30 cycle 95 ºC 30 sec, 55 ºC 30 sec, 72 ºC 2 min, and 72 ºC 10 min). The longer 3' end region containing the single nucleotide point mutations were amplified using primers 421 and 43 in the case of MP/S120A, and primers 422 and 43 in the case of MP/S120D (PCR conditions: 95 ºC 5 min, 30 cycle 95 ºC 30 sec, 55 ºC 30 sec, 72 ºC 6 min, and 72 ºC 10 min). The two amplified regions were connected with overlap PCR using primers 57 and 43 (PCR conditions: 95 ºC 5 min, 30 cycle 95 ºC 30 sec, 60 ºC 30 sec, 72 ºC 1 min, and 72 ºC 10 min). The PCR products were separated on 1% agarose gel and was isolated using High-Pure Roche Purification Kit (Roche). After the isolation and purification, the PCR products were ligated into pGEM® T-Easy plasmid and was transformed into *E. coli* DH5α. Minipreps were prepared from the bacterial colonies and the presence of the insert was tested using restriction enzyme *Eco*RI, while the presence of the mutation was tested using *Bgl*II and *Sac*I. The sequences of the mutant clones were checked by nucleotide sequence determination (Biomi Kft., Gödöllő).

Table 1. The nucleotide sequene of the oligonucleotide primers used. The restriction enzymes are marked in italics and the codons of the point mutations are in bold.

Ref. number	Name	Nukleotide sequence (5'-3')
57	CMV RNA3 for	GG*CTGCAG*TAATACGACTCACTATAGTAATCTTACCAC
418	MP/S28A for	GG*AGATCT*TATT**TGC**CCCTGAAGCCATTAAGAAAATGGC
419	MP/S28D for	GG*AGATCT*TATT**TGA**CCCTGAAGCCATTAAGAAAATGGC
420	MP/S28AD rev	GG*AGATCT*TTTGAAGATCGTCAGACGTATCCGCTGAGG
421	MP/S120A for	GG*GAGCTC*G**CTC**CCATAGATGGGCAATGCGTTTCG
422	MP/S120D for	GG*GAGCTC*G**ATC**CCATAGATGGGCAATGCGTTTCG
423	MP/S120AD rev	GG*GAGCTC*CTTGTCGCCTAGATCAGCTAAGTAAATTCTCAA
43	CMV uni 3' rev	GCC*GGATCC*CTAAAGACCGTTAACCACCTGC
463	MP SacI for	GG*GAGCTC*ATGGCTTTCCAAGGTACCAGT
464	MP-eGFP rev	CGCCCTTGCTCACCATAAGACCGTTAACCACCTGC
465	MP-eGFP for	GTGGTTAACGGTCTTATGGTGAGCAAGGGCGAGG
466	eGFP BamHI rev	CC*GGATCC*TCACTTGTACAGCTCGTCCATG
493	CMVMP pET for	GG*CATATG*GCTTTCCAAGGTACCAGTAG

273	CMVMP pET rev	GCC*GGATCC*CTAAAGACCGTTAACCACCTGC
AktinF	Cap. aktin for	AGGGATGGGTCAAAAGGATGC
AktinR	Cap. aktin rev	GAGACAACACCGCCTGAATAGC

4.2.2.2. Creating wild type and mutant clones for protein expression

The wild type and mutant movement proteins were amplified using PCR with primers 493 and 273 (Table 1). The pRs3 and MP/S28A infectious clones were used as templates. The PCR was performed using the following conditions: primary denaturation 95 ºC 5 min, 30 cycle of 95 ºC 30 sec, 50 ºC 30 sec, 72 ºC 3 min, and a final elongation step of 72 ºC 10 min. The PCR products were separated on 1% agarose gel and was isolated using High-Pure Roche Purification Kit (Roche). After the isolation and purification, the PCR products were ligated into pGEM® T-Easy plasmid and were subcloned into pET28 expression plasmid using restriction enzymes *Nde*I and *Bam*HI, and were transformed into *E. coli* DH5α. After miniprep preparation the MP-containing plasmids were transformed into *E. coli* BL21DE3 (Rs-CMV MP pET, MP/S28A pET). Miniprep was prepared from the bacterial colonies and the presence of the insert was tested using restriction enzymes *Nde*I and *Bam*HI. The sequences of the mutant clones were checked by nucleotide sequence determination (Biomi Kft., Gödöllő).

4.2.2.3. Creating wild type and mutant clones for the subcellular localization assay

For the subcellular localization assay, GFP was fused to the C-terminal end of the wild type and mutant MPs using overlap PCR. The previously created mutant clones were used as templates (pRs3, MP/S28A, MP/S28D). A two-step PCR method was used when creating the GFP-fused clones (CMV MP-eGFP, MP/S28A-eGFP, MP/S28D-eGFP). First, the 5’ end region of CMV RNA3 clone (which contains the 5’ non-coding region and the MP) was amplified using primers 463 and 464 (PCR conditions: 95 ºC 5 min, 30 cycle 95 ºC 30 sec, 60 ºC 30 sec, 72 ºC 1 min, and 72 ºC 10 min), and the GFP was amplified from eGFP clone using primers 465 and 466 (PCR conditions: 95 ºC 5 min, 30 cycle 95 ºC 30 sec, 60 ºC 30 sec, 72 ºC

1 min, and 72 ºC 10 min). The two PCR product was connected using overlap PCR with primers 463 and 466 (PCR conditions: 95 ºC 5 min, 30 cycle 95 ºC 30 sec, 65 ºC 30 sec, 72 ºC 2 min, and 72 ºC 10 min). The resulting 1569 nt long PCR products were separated on 1% agarose gel and was isolated using High-Pure Roche Purification Kit. After the isolation and purification, the PCR products were ligated into pGEM® T-Easy plasmid and was transformed into *E. coli* DH5α. Minipreps were prepared from the bacterial colonies and the presence of the insert was tested using restriction enzyme *Eco*RI. The sequences of the mutant clones were checked by nucleotide sequence determination (Biomi Kft., Gödöllő). The clones were cloned into pBin61 binary vector using restriction enzymes *Sac*I and *Bam*HI and were transformed into *E. coli* again. New minipreps were prepared and were checked with restriction enzymes. After that, they were transformed into *A. tumefaciens* C53C1 and the minipreps were checked with restriction enzymes one more time.

4.2.3. Protein expression in *E. coli* BL21DE3, Western blot

The clones (Rs-CMV MP pET, MP/S28A pET) were incubated in 50 mL liquid LB media at 37 ºC, until the optical density (OD_{600}) of the bacterial solution reached the value of 0.75. After adding 100 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) the bacterial suspension was incubated at 37 ºC for 2 hours. The bacterium suspension was eliquoted into 1–1 ml, and after centrifugation, the bacterial pellet was resuspended in 50 µL Laemmli buffer (62.5 mM Tris-HCl pH: 6.8, 2.5% SDS, 0.002% bromophenol blue, 5% β-mercaptoethanol, 10% glycerol), and was heated to 100 °C.

The phosphorylation of the expressed proteins was investigated with Western blot. After denaturation (boiling for 5 min), the proteins were separated with SDS-containing 12% polyacrylamide gel electrophoresis (80 V, 150 min), and were transferred to a nitrocellulose membrane (GE Healthcare Bio-Sciences) (200 mA, 90 min). During the immunoblot reaction, Western Blocker Solution (Sigma) was used for the blocking step, anti-phosphoserine IgG primary antibody (Qiagen) and anti-mouse HRP conjugated IgG secondary antibody (Agrisera)

were used. For the chemiluminescent detection, Pierce™ ECL Western Blotting Substrate (Thermo Scientific) was used.

4.2.4. *In vitro* RNA transcription, plant inoculation and virion purification

The wild type and mutant infectious clones, together with the RNA1 and RNA2 clones (pRs1, pRs2, pRs3, MP/S28A, MP/S28D, MP/S120A, MP/S120D) were linearised with *Bam*HI restriction enzyme. 50 u T7 RNA polymerase was used for the RNA transcription (SZILASSY és mtsai., 1999) with 1 µg linearized template, 50 mM ATP, UTP and CTP-t, 6.25 mM GTP, 50 mM CAP (7-metil-guanosine cap) and 50 u RiboLock RNase-inhibitor. After incubation for 15 min at 37 ºC, 25 mM GTP was added to the samples, and they were incubated at 37 ºC for an hour. The result of the RNA transcription was checked with 1% agarose gel electrophoresis.

N. benthamiana plants were mechanically inoculated using equal amounts of RNA1, RNA2 and wild type or mutant RNA3 *in vitro* transcripts. Carborundum-containing inoculation buffer was added to the mixture (25 mM glycine, 15 mM K_2HPO_4, 0,5% bentonit, 0,5% cellite), and the 3-4 week old plants were mechanically inoculated.

Three weeks after the inoculation virions were purified from the symptomatic young leaves of the inoculated according to the method of Lot et al. (1972). 100 g plant material was homogenized in 1:1 ration of Na_3-citrate buffer (0.5 M Na_3-citrate (pH: 6.5), 0.1% thioglycolic acid, 0.5 M EDTA (pH: 8) and chloroform. After centrifugation (4 ºC, 10 min, 1890 RCF) 10% PEG 6000 was added to the supernatant, and after 15 min stirring, 30 min incubation and centrifugation (4 ºC, 30 min, 5750 RCF), the pellet was resuspended in 0.5 mM borate buffer (pH: 9) and 2% Triton X solution. After centrifugation (4 ºC, 10 min, 5750 RCF) the sample was ultracentrifuged in borate buffer and Triton X solution for 2.5 hours on 85 000 RCF at 4 ºC. The pellets were resuspended in borate buffer.

4.2.5. Inoculation of test plants

Purified virions were used for the test plant inoculation assays with the concentration of 10 μg/mL. The mechanical inoculation was performed using inoculation buffer (25 mM glycine, 15 mM K_2HPO_4, 0,5% bentonit, 0,5% cellite) and carborundum. The *N. tabacum* cv. Xanthi-nc and *C. murale* plants were inoculated in 4-weeks-old state, while the cotyledons of cucumber were inoculated before the appearance of the young leaves. The plants were all grown in long day conditions and were monitored for 5 weeks. The negative control plants were mock inoculated with inoculation buffer and carborundum.

4.2.6. RNA extraction, cDNS preparation and RT-PCR

To verify the stability of the mutations, total RNA was extracted from the purified virions and from the upper non-inoculated leaves of *N. benthamiana*, *N. tabacum* cv. Xanthi-nc and *C. sativus* with SV Total RNA Isolation Kit (Promega) according to the manufacturer's instructions. RT-PCR was carried out using primers amplifying the full-length ORF of CMV MP (Table 1). Prior DNA sequence determination the PCR products were purified with High Pure PCR Product Purification Kit (Roche).

4.2.7. Western blot, press blot

Protein extracts from *N. tabacum* cv. Xanthi-nc were prepared from 20 mg leaf samples homogenized in Laemmli-buffer. Prior SDS-PAGE, the samples were denatured at 95 ºC for 5 min. After 1 min cooling on ice, the samples were briefly centrifuged. The samples (1-10 μL) were separated on 12% acrylamide gel. The equivalence of the proteins was verified using Coomassie Brilliant Blue Staining G250. After electrophoresis (80 V, 150 min) proteins were transferred to a nitrocellulose membrane (200 mA, kb. 90 min) and were hybridized using anti-CMV CP primary antibody and anti-rabbit ALP conjugated IgG (Agrisera). For the detection AP Conjugate Substrate Kit (Bio-Rad) was used.

For the tissue blot immunoassay, the cucumber cotyledons were collected 10 days after inoculation. The surfaces of the cotyledons were gently cut and the leaves were placed on a nitrocellulose membrane. By applying pressure to the cut leaves, the plant sap was pressed on onto the membrane. After drying the membrane, it was hybridized by anti-CMV CP primary antibody and anti-rabbit ALP conjugated secondary antibody. For the detection AP Conjugate Substrate Kit was used.

4.2.8. Analysis of the local lesions on *C. murale*

In case of *Ch. murale* the inoculated leaves were collected 3 days post inoculation. The data regarding the size of the necrotic lesions were processed using Image J software (version 1.52, NIH). Data were analyzed by Kolmogorov–Smirnov test, skewness and kurtosis for normality of the data, and Levene's test for homogeneity of variances. To evaluate the differences between the areas of the induced local necrotic lesions one-way ANOVA model coupled with Games-Howell's post hoc test were used.

4.2.9. *Agrobacterium*-mediated transient gene expression

The bacterium suspensions were incubated and shaked in liquid LB media containing rifampicin and kanamycin for 16 hours at 28 ºC. After centrifugation, 0.01 M $MgCl_2$ and acetosyringone-containing MES buffer was added to the bacterial pellet. The optical density of the samples was measured (OD_{600}), and the bacterial suspensions were mixed with the following concentrations: the P19 silencing suppressor protein (JAY és mtsai., 2023), which was added to all the samples was used with 0.2, and the GFP-fused wild type and mutant MPs were used with 0.4 OD_{600} value. The mixed bacterial suspensions were infiltrated into *N. benthamiana* and *N. tabacum* cv. Xanthi-nc leaves using a needle-less syringe.

4.2.10. Confocal laser-scanning electron microscopy

Images were obtained using a Leica TCS SP8 confocal laser-scanning microscope (Leica Microsystems GmbH). Images were acquired by a HC PL APO CS2 40×/1.10 water

immersion objective. Confocal aperture size was set to 0.585 Airy Unit. Image acquisition was carried out by bidirectional scanning along the x-axis, and images were averaged from three distinct image frames in order to reduce image noise. Aniline blue dye was excited at 405 nm, staining pattern was detected between 410–480 nm. GFP was excited at 488 nm, the emitted fluorescence was detected between 490–530 nm.

Callose at PD was stained with aniline blue (2:3 ratio of 0.1% aniline blue and 1 M glycine pH: 9.5) by infiltration into leaves 10 min before visualization. For plasmolysis, leaf sections were incubated in 10% NaCl and the plasmolyzed cells were examined by confocal microscopy.

3. RESULTS

3.1. *In silico* analysis of CMV MP

In the case of CMV MP, there is no available data on how various post-translational modifications (including the most commonly occurring phosphorylation) affect the protein's function. First, we identified potential phosphorylation sites in the amino acid sequence of the protein using online phosphorylation site prediction programs. The NetPhos 3.1 software identified numerous amino acids, among which were 21 Serines, 12 Threonines, and 6 Tyrosines. For CMV MP, we selected two serine amino acids, Ser28 and Ser120, which were identified as the most likely phosphorylation sites, located at positions 28 and 120.

3.2. Phosphorylation of CMV MP in *E. coli*

Based on the *in silico* analysis, we hypothesized that Ser28 and Ser120 are phosphorylatable. We investigated this hypothesis with expressing the wild type Rs-CMV MP and the S28A-containing mutant MP in *E. coli*. The bacterial suspensions were grown in liquid media until they reached 0.75 OD_{600} value. After adding IPTG we incubated the suspensions for 2 hours at 37 ºC.

We examined the phosphorylation of the wild-type and MP/S28A proteins using Western blot. Through hybridization with the phosphoserine antibody, we determined that the MP/S28A protein (where the point mutation leads to a stable non-phosphorylated state at amino acid 28) showed significantly lower levels of phosphorylation compared to the wild-type MP. Coomassie staining confirmed that there was no substantial difference in the expressed amounts of the two proteins, thus supporting that the significant difference observed in phosphorylation levels cannot be attributed to differences in protein quantities.

Based on these results, we have demonstrated that the Ser28 amino acid is indeed phosphorylated in the CMV MP expressed in *E. coli* bacteria. This validates the relevance of the phosphorylation sites predicted by the NetPhos 3.1 program under *in vivo* conditions.

3.3. Inoculation with transcripts of Rs-CMV, MP/S28A, MP/S28D, MP/S120A, MP/S120D, virion purification

In order to investigate the role of phosphorylation at positions Ser28 and Ser120 in virus infection, we replaced the serine amino acid with alanine and aspartic acid at both positions. Alanine is a small, non-polar amino acid incapable of phosphorylation, effectively modeling the non-phosphorylated state (MP/S28A, MP/S120A). On the other hand, aspartic acid, due to its negative charge and side chain size, is ideal for modeling the phosphorylated state (MP/S28D, MP/S120D). These point mutations were introduced into the infectious clone of the RNA3 of the Rs-CMV strain. For control infections, we used the wild-type RNA3 clone.

The infectivity of the generated constructs was tested on *N. benthamiana* plants. Transcripts were prepared from the clones, which were used for plant inoculation along with a carborundum-containing inoculation buffer, and transcripts of RNA1 and RNA2. Fourteen days after infection, we observed leaf deformation and mosaic leaf symptoms for each clone, providing evidence of the constructs' infectivity.

Using RT-PCR, we detected the presence of the virus in the samples. By determining the nucleotide sequence of the PCR product, we confirmed that the mutations remained stable two weeks after inoculation. We purified virions from 100 g of plant samples for further host plant investigations.

3.4. Characterization of local and systemic symptoms induced by wild type and mutant CMV

3.4.1. Investigation of systemic symptoms induced by MP/S28A, MP/S28D, MP/S120A and MP/S120D and Rs-CMV on *N. tabacum* cv. Xanthi-nc

We inoculated *Nicotiana tabacum* cv. Xanthi-nc test plants with wild-type and mutant CMV virions (Rs-CMV, MP/S28A, MP/S28D, MP/S120A, MP/S120D) to investigate differences in systemic symptoms. In tobacco plants infected with Rs-CMV, four days after

inoculation (dpi), we observed systemic mosaic and mild leaf deformation. The MP/S120A and MP/S120D mutants caused similar symptoms, but they appeared later than in the case of wild-type CMV. Plants infected with MP/S28A and MP/S28D mutants exhibited much milder symptoms. On the fifth day, when wild-type CMV and mutants modified at the 120th amino acid position already displayed characteristic systemic symptoms, plants infected with MP/S28A and MP/S28D mutants only showed vein clearing on young leaves.

The differences in symptom formation were further supported by molecular analyses. In the case of Rs-CMV, the virus was detectable in large quantities in young, non-inoculated leaves as early as three days after infection. For the MP/S120A and MP/S120D mutants, although virus presence was detectable in infected plants three dpi, the level of virus accumulation characteristic of wild-type CMV was only reached one day later, on 4 dpi. In the case of MP/S28A and MP/S28D, virus accumulation was observed even later, on the fifth day. The stability of the mutations was confirmed by RT-PCR performed 10 dpi, followed by nucleotide sequence analysis. Based on our experiments, we determined that the MP/S28A and MP/S28D mutants, which resulted in milder symptoms compared to the wild type, exhibited significantly slower virus accumulation, aligning with the observed differences in symptom development.

3.4.2. Investigation of the systemic symptoms induced by MP/S28A, MP/S28D, MP/S120A and MP/S120D in cucumber (*C. sativus*)

We also examined the development of systemic symptoms in cucumber (*C. sativus*). Ten dpi plants infected with wild-type Rs-CMV exhibited large necrotic lesions on the inoculated cotyledons, and systemic mosaic and leaf deformation on their young leaves. Cucumbers infected with the MP/S120A and MP/S120D mutants showed symptoms similar to those induced by Rs-CMV, with slightly smaller necrotic lesions on the cotyledons. In the case of the 28th amino acid mutants (MP/S28A and MP/S28D), neither local nor systemic symptoms were observed.

We supported these differences with molecular methods as well. From the inoculated cotyledons, we conducted press blot hybridization, and from the non-inoculated young leaves, we used Western blot and RT-PCR to examine the presence of the virus. Hybridization was performed with CMV CP primary antibodies in both press blot and Western blot. In the RT-PCR, a section of the gene encoding CP was amplified, using primers targeting an actin gene. We observed that while Rs-CMV and the MP/S120A and MP/S120D mutants were detectable in both the inoculated cotyledons and systemic leaves, the MP/S28A and MP/S28D mutants were not detectable in the plant at all.

3.4.3. Investigation of the local necrotic symptoms induced by MP/S28A, MP/S28D, MP/S120A and MP/S120D in *C. murale*

We examined the symptomatic differences between wild-type and mutant CMVs on the local lesion host plant of CMV (*C. murale*). On 4 dpi, plants infected with wild-type CMV exhibited large local lesions on the inoculated leaves, similar to those infected with the 120th amino acid mutants (MP/S120A, MP/S120D). However, leaves inoculated with MP/S28A and MP/S28D mutants showed only small punctate lesions. To confirm the difference in lesion size, we quantified and statistically analyzed the area of lesions formed on the inoculated leaves. This analysis revealed significant differences in the symptoms induced by the viruses ($p<0.0001$). The size of lesions caused by the MP/S120D mutant did not significantly differ from those caused by the wild type. The areas of lesions induced by MP/S120A, MP/S28A, and MP/S28D mutants were significantly different from each other, as well as from the wild type, on the inoculated leaves. This statistical analysis supports our visual observations.

3.5. Investigation of the wild type and mutant CMV MP localization

3.5.1. The effect of CMV MP Ser28 mutations on PD localization

During our host plant experiments, the most significant difference was observed with the mutation of the CMV MP Ser28 amino acid. Therefore, we examined whether the S28A and S28D point mutations affected the intracellular localization of MP. The primary function of MP is to facilitate the cell-to-cell spread of the virus, thus the wild-type MP mainly localizes near the plasmodesmata (NAVARRO és mtsai., 2019). We fused GFP to the C-terminus of both the wild-type (Rs-CMV) and the mutant (S28A and S28D) MP. Using confocal laser scanning microscopy, we investigated the intracellular localization of the proteins. Microscopic images of the epidermal cells of *N. benthamiana* and *N. tabacum* cv. Xanthi-nc leaves were taken 24-48 hours after agroinfiltration. The GFP-fused wild-type MP (MP-eGFP) was observed along the cell wall in small punctate formations in both plants. After staining the infiltrated tissues with aniline blue (which made the plasmodesmata visible), there was a significant overlap between the green fluorescent spots indicating MP localization and the blue fluorescent spots used as PD markers. This observation aligns with the previous observations (BLACKMAN és mtsai., 1998; DING és mtsai., 1995; ITAYA és mtsai., 1997). In the case of the GFP-fused mutant MP constructs (MP/S28A-eGFP, MP/S28D-eGFP), we also observed MP-PD colocalization, but to a significantly lesser extent compared to the wild-type MP-eGFP.

3.5.2. The effect of CMV MP Ser28 mutations on PD localization in plasmolyzed cells

We also conducted the experiment on plasmolyzed cells of *N. tabacum* cv. Xanthi-nc. During plasmolysis, the cell wall and the plasma membrane separate, allowing a better observation of plasmodesma localization. Following agroinfiltration and staining with aniline blue, the cells were immersed in a 10% NaCl solution, and images were captured using confocal laser scanning microscopy. We observed that in the case of MP/S28A-eGFP and MP/S28D-eGFP mutants, the mutant MPs, along with the shrunken plasma membrane, moved away from the cell wall and no longer colocalized with the PD. In contrast, the wild-type MP-eGFP, despite plasmolysis, remained positioned along the cell wall, still associated with the PD.

4. DISCUSSION

The phosphorylation of viral MPs has been confirmed in several cases (LEE és LUCAS, 2001). In the case of CMV MP, there is only one available data where the presence of phosphorylation was confirmed in transgenic tobacco plants, but the exact location and function of the phosphorylation were not investigated (MATSUSHITA és mtsai., 2002). We aimed to examine the role of CMV MP phosphorylation in the virus lifecycle. In order to do this, we conducted *in silico* analysis and identified Ser amino acids at positions 28 and 120 of CMV MP as potential phosphorylation sites. Using PCR-based methods, we modified these amino acids and introduced point mutations, thus modeling the non-phosphorylated (Ala) and phosphorylated (Asp) states.

So far the impact of MP phosphorylation on symptom development has been reported in only one case. In the case of *Abutilon mosaic virus* (AbMV), when infecting tobacco plants (*N. benthamiana*), *Malva parviflora*, *Datura stramonium* and *Nicandra physaloides* with Ala and Asp mutants of three phosphorylatable amino acids (Thr221, Ser223, Ser250), the symptoms development was altered. (KLEINOW és mtsai., 2009, 2020).

To investigate the role of Ser28 and Ser120 phosphorylation during CMV infection, we infected various test plants and observed the differences in symptoms caused by the wild-type (Rs-CMV) and mutant viruses (MP/S28A, MP/S28D, MP/S120A, MP/S120D). The results revealed significant differences in symptoms and symptom development dynamics between the 28th amino acid mutants (MP/S28A, MP/S28D) and the wild type. When infecting *N. tabacum* cv. Xanthi-nc plants, we observed a significant difference in the pace of systemic symptom appearance. Plants infected with MP/S28A and MP/S28D mutants only reached the level of virus accumulation on the 5th day post inoculation (dpi), which the wild-type Rs-CMV exhibited on 3 dpi. On a local lesion host plant for CMV (*C. murale*), MP/S28A and MP/S28D mutants caused small, punctate lesions on the inoculated leaves in contrast to the larger lesions observed with the wild type. On a commercially significant host plant, cucumber, the 28

mutants were unable to infect the cucumber at all, and the presence of the virus could not be detected in either young leaves or inoculated cotyledons.

The mutants targeting the amino acid Ser120 (MP/S120A, MP/S120D) showed slightly less differences compared to the symptoms induced by Rs-CMV. On the 4th day post inoculation in *N. tabacum* cv. Xanthi-nc, we detected similar amounts of virus concentrations of Rs-CMV present on 3 dpi. In the case of *C. murale* infection, the size of the lesions observed on the inoculated leaves significantly differed only in the case of MP/S120A. No significant difference in symptom development was observed between MP/S120A, MP/S120D, and Rs-CMV on cucumber.

One of the main function of MPs is to facilitate the cell-to-cell movement of the virus genome through PD. The relationship between phosphorylation and the intracellular localization of viral MPs has already been demonstrated in some cases. Among tobamoviruses, such as *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV), it was demonstrated that phosphorylation is responsible for MP PD localization. When the phosphorylatable amino acids were substituted with Ala or Asp, PD localization ceased or significantly deteriorated, negatively impacting the virus's spread. (KAWAKAMI és mtsai., 1999; TRUTNYEVA és mtsai., 2005). The role of phosphorylation in PD localization has also been confirmed in the case of *Potato leafroll virus* (PLRV). Furthermore, it has raised the possibility of successive phosphorylation, suggesting the phosphorylation of two serines are primarily required, and only after this does the phosphorylation of another two serine amino acids occur, which facilitates the breakdown of the virus ribonucleoprotein complex and inhibits cell-to-cell movement. This means that the phosphorylation of one amino acid (and its "on-and-off" role) may not be sufficient to alter a protein's function. Instead, a more complex, possibly multiplayer process needs to occur in the appropriate sequence for the change to take place.

We examined whether the phosphorylation of Ser28 affects the intracellular localization of CMV MP, as the MP/S28A and MP/S28D virus mutants had the most impact on CMV symptom development. In line with previous results, our experiments showed that the wild-

type CMV MP-eGFP localized along the cell wall, at the plasmodesmata. In contrast, the intracellular localization of MP/S28A-eGFP and MP/S28D-eGFP mutants localized differently. The mutants were localized dispersed along the cell wall in a punctate pattern, rather than co-localizing with aniline blue-stained callose. Plasmolysis experiments confirmed that the mutants were localized in the plasma membrane, rather than being associated with PD. These findings suggest the possibility that phosphorylation of the Ser28 amino acid influences the MP PD localization. Based on our results, the intracellular localization of mutants containing both Ala and Asp was altered, indicating that Ser28 phosphorylation does not exhibit an "on-and-off" switch effect on the protein's function. Instead, it suggests that dynamic oscillation between phosphorylated and non-phosphorylated states plays a crucial role in proper intracellular localization.

Our results shed some light on a previously unknown mechanism in the process of CMV MP PD localization. Ser28 is conserved among members of the Cucumovirus genus, and its Ala and Asp mutants caused detectable differences in CMV symptom development in both systemic and local host plants. This confirms the significant role of a new, previously unidentified phosphorylation site in CMV MP, influencing the intracellular localization of the CMV MP with PD.

5. NEW SCIENTIFIC RESULTS

1. We identified amino acids at positions 28 and 120 of CMV MP as potentially phosphorylatable through *in silico* analysis, and we demonstrated their role in the intracellular localization of the movement protein and symptom development.

2. We confirmed the phosphorylation of the Ser28 amino acid in CMV MP expressed in *E. coli*.

3. By replacing Ser28 with alanine and aspartic acid (modeling the non-phosphorylated and phosphorylated states), we observed that the mutant viruses (MP/S28A, MP/S28D) developed mosaic symptoms much slower on the upper, non-inoculated leaves of *Nicotiana tabacum* cv. Xanthi-nc, and induced significantly smaller lesions on the inoculated leaves of *Chenopodium murale*, and were not detectable in the inoculated cotyledons and the young leaves of cucumber.

4. We found that the mutants with alanine and aspartic acid substitutions at position Ser120 induced mosaic symptoms on the upper, systemic leaves of *N. tabacum* cv. Xanthi-nc later than the wild type Rs-CMV. On the inoculated leaves of *C. murale*, MP/S120A caused significantly smaller lesions than Rs-CMV.

5. We demonstrated that altering Ser28 has a significant impact on the intracellular PD localization of CMV MP. The mutants were significantly less able to associate with PD; instead, they were located along the cell wall, within the plasma membrane.

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7. PUBLICATIONS RELATED TO THE DISSERTATION SUBJECT

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