Szent István University
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EFFECTS OF NON-HOMOLOGOUS MOVEMENT PROTEIN ON THE MOVEMENT AND SYMPTOMATOLOGY OF CUCUMBER MOSAIC VIRUS

EMESE HUPPERT

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Program: Biology PhD Program

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Program leader: Dr. Zoltán Tuba
Professor, Head of department
Szent István University,
Faculty of Agricultural and Environmental Sciences,
Department of Botany and Plant Physiology

Supervisor: Dr. Ervin Balázs
MHAS, Director
Agricultural Biotechnology Center,
Institute of Environmental Biosafety

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Dr. Zoltán Tuba
program leader

Dr. Ervin Balázs
supervisor

INTRODUCTION

Cucumber mosaic virus (CMV) adapts efficiently to different environmental conditions and is known to be one of the most important agricultural pathogens. CMV has exceptionally large host-range composed of more than thousand plant species (Edwardson and Christie, 1991). Defense against CMV is of utmost importance in plant protection due to its agricultural significance. In order to efficiently defend plants from CMV, it is essential to know its structure, life cycle and mechanism.

Since both the virus and the host plant participate actively in shaping the interaction during the infection, it is impossible to study the virus functions separately from the impacts of the host-plants. When a virus gets into a plant, the pathogen confronts the host plant organism; the former is trying to establish infection, the latter is aiming at preventing this process. In order to infect a host plant, viruses have to be able to (i) replicate in the cells of the host; (ii) get to the neighboring cell from the originally infected one (short distance movement) and (iii) spread systemically in the plant through its phloem (long distance movement) (Carrington et al., 1996). Short distance movement is crucial for the outcome of the virus infection. A virus lacking the capacity to move is confined to the initially infected cells and unable to get to the phloem and cause systemic symptoms on the plant.

Most of the plant viruses encode one or more special movement proteins (MP) that are indispensable for virus movement. Besides the movement protein, the coat protein (CP) also plays important role in the local movement of CMV. In absence of CP, *Cucumber mosaic virus* is not able to move from cell to cell (for review see Palukaitis and García Arenal, 2003). On the contrary *Cymbidium ringspot virus* (CymRSV) does not require the presence of the coat protein for its local movement (Dalmay et al., 1993). According to the current data, both

viruses use the so-called ’TMV like’ movement mechanism typical for the *Tobacco mosaic virus* local movement (see Hull, 2002).

Use of pseudorecombinant and hybrid viruses to examine different virus genes is a popular and effective method of molecular virology. However, the ability of the foreign gene or gene product to interact and efficiently cooperate with the other proteins of the examined virus is a basic precondition for such experimental systems.

In our laboratory we are studying the movement of CMV and the interactions between the virus and different host plants. Utilizing the above mentioned fact, namely that contrary to CymRSV the CMV requires the presence of its CP for short distance movement, we aimed at examining the infectivity of a hybrid *Cucumber mosaic virus* carrying the movement protein gene of the *Cymbidium ringspot virus*.

This aim implied the following objectives:

- Construction of a hybrid CMV with the MP gene of CymRSV;
- Mapping of infectivity of the hybrid virus on different host plants;
- Examination of the movement of the hybrid virus regarding its requirement for the CP.

MATERIALS AND METHODS

Virus strains

- Trk7-CMV strain (Beczner et al., 1978). The EMBL/GenBank/DDBJ accession numbers of RNA 1, RNA 2 and RNA 3 are AJ007933, Aj007934 and L15336, respectively (Salánki et al., 1994; Szilassy et al., 1999).
- CymRSV (Hollings et al., 1977; Burgyán et al., 1990; accession number: NC003532).

Host plants

Nicotiana glutinosa L, *N. megalosiphon* Van Heurck&Mull. Arg., *N. tabacum* L. cv. Xanthi, *N. debneyi* Domin., *N. benthamiana* Domin., *N. clevelandii* Gray., *Chenopodium quinoa* Wildd., *C. murale* L. and *C. foetidum* L. plants were used for the experiments. The plants were kept in nursery chambers (10 hours in dark on 18°C/14 hours in light on 23°C).

Bacteria strains

The cDNA clones were maintained and propagated in *Escherichia coli* DH5-α, GM2163 and TG90 strains.

Construction of chimeric and mutant cDNA clones

The cDNA clone of Trk7-CMV RNA3 (pCMV3; Salánki et al, 1994) was used for constructing the pCMV3cymMP chimeric clone. For amplification of the CymRSV movement protein gene the pCym19STOP clone (courtesy of Dr. Burgyán József) was used as a template. The 5' and 3' primers used for the PCR reaction contained an SpeI restriction endonuclease (5'-CGACTAGTCATGGACACTGAATACCAAC-3') and an AgeI restriction endonuclease (5'-GCACCGGTCTAGACTGAAGAGTCTGTCC-3'), respectively. The parameters of the PCR reaction were as follows: 30 sec denaturation on 94°C, 30 sec anellation on 54°C and 50 sec synthesis on 72°C. The obtained PCR product was introduced into the adequate XbaI-HpaI restriction endonuclease site of pCMV3.

For construction of the coat protein defective mutants, the pCMV3 and pCMV3cymMP clones were digested with AgeI and Tth111I restriction endonucleases. The flanking ends were filled up by Klenow I fragment of DNA polymerase I, and the plasmids were self-ligated. As result, the 3'-end 210 nucleotides are absent from the obtained pCMV3ΔCP and pCMVcymMPΔCP clones.

In ΔCP-GFP version of pCMV3 clone (pCMV3ΔCP-GFP), the whole CP gene is replaced with the green fluorescent protein (GFP) gene. To achieve this we introduced an NdeI restriction endonuclease site before the CP gene as described by Salánki et al. (1997). The GFP gene was amplified from TU#60 clone with M13 -28 3' and GFP/NdeI (CGCATATG*AGTAAAGGAG*) 5' primers. The parameters of the PCR reaction were as follows: 20 sec denaturation on 94ºC, 40 sec anellation on 45º, 1 min synthesis on 72ºC. The obtained PCR product was digested with NdeI and EcoRI restriction endonucleases and introduced to the NdeI-EcoRI site of pCMV3 CP gene.

The CP gene of the CMV RNA3 construct carrying the MP gene of CymRSV was also replaced by GFP gene resulting in pCMV3-cymΔCP-GFP. To obtain this construct, the BamHI-ApaI fragment (the 5' half of the clone) of pCMV3-cymΔCP construct was ligated into the appropriate BamHI-ApaI location of pCMV3ΔCP-GFP.

Sequencing

The nucleotide sequence of the CMVcymMP clone 5'-end was determined by automatic fluorescent stop nucleotide method (Applied Biosystems Gene Analyzer 3100).

In vitro RNA transcription

The *in vitro* transcription reaction mix (50 µl final volume) contained 1 µg template, 50 mM ATP, CTP, UTP and 6,25 mM GTP, 50 mM m^7GpppG CAP, 50 u T7 RNS-polymerase, 50 u RNAase inhibitor, 5 mM ditiothreitol, 3 mM MgCl, 2 mM spermidin and 20 mM Tris-HCL. The reaction mix was incubated for 60 minutes on 37°C.

Infection of plants

The inoculum used for infection of the plants contained equal amount of *in vitro* RNA transcripts corresponding to RNA1, RNA2 and RNA3 of CMV. The mixture of *in vitro* transcripts was diluted to 1:1 ratio with 50 mM Na-phosphate buffer (pH=8,6). To

present the symptoms of CMV and CymRSV the tissue sap of infected plants was diluted with Na-phosphate buffer and used as inoculum. The plants were inoculated mechanically.

Analysis of the RNA accumulation

The 1 cm diameter samples were collected from the leaves 6 days (inoculated leaves) and 14-20 days (systemic leaves) after the inoculation. The nucleic acid was extracted according to White and Kaper (1989). Northern blot analysis was carried out according to Sambrook et al. (1989). Random primer method (HexaLabel Kit, Fermentas) was used to produce the ^{32}P labeled radioactive probes. The radioactive probes employed during the experiments were specific to RNA3 of CMV and the MP gene of CymRSV.

Virus purification

The virus was propagated in *Nicotiana benthamiana* plants. Virions were extracted from the infected plants 14 days after the inoculation, with the method of Lot et al. (1972). Phenol-chloroform extraction was used to obtain RNA.

Detection of GFP fluorescence

For GFP fluorescence detection, the plants were illuminated with 100-W long-wavelength UV lamp (Blak Ray; model B100AP; UV Products). Minolta SRT MC-II camera, Kodak Elite Chrome II film (200 ASA for macroscopic and 400 ASA for microscopic shots) and Canon Y1 filter were used for taking the pictures, with the following parameters: exposition: 20 sec; distance: 0,8 m; blend: f/8. The microscopic shots were taken with Olympus IMT-2 microscope, employing BP405 and Y455 filters.

RESULTS

A hybrid *Cucumber mosaic virus* was constructed. The hybrid CMV virus (CMVcymMP) contains the MP gene of *Cymbidium ringspot virus* instead of the CMV MP gene. The hybrid virus proved to be viable and infected efficiently the examined host plants.

Nine plant species (*Chenopodium murale*, *C. quinoa C. foetidum*, *Nicotiana benthamiana*, *N. clevelandii*, *N. megalosiphon*, *N. debneyi*, *N. glutinosa*, *N. tabacum* cv. Xanthi) were employed to examine the infection characteristics of the hybrid virus.

- The C*henopodium* species are local hosts of both parental viruses (CMV and CymRSV). The hybrid virus caused local lesions on these plants. The size and morphology of the local lesions were determined by the foreign MP gene.
- The group II of the examined plant species consisted of tobacco plants that are systemic hosts of both parental viruses (*N. benthamiana, N. clevelandii* and *N. megalosiphon*). Similarly to CMV and CymRSV the hybrid virus also infected these plants systemically. The symptoms of CMVcymMP infection showed similarity to the symptoms of both CMV and CymRSV.
- The CMV infects systemically whereas the CymRSV infects only locally the plants assigned to the group III of the examined plants. The hybrid virus behaved differently on these host species depending on the given plant: CMVcymMP infected systemically *N. debneyi* and *N. tabacum* cv. Xanthi, however induced only local lesions on *N. glutinosa*.

210 nucleotides were removed from the 3'-end of the CP gene to construct the CP deficient mutants of CMV and CMVcymMP. The deletion lead to loss of movement function in case of CMV. The CMVcymMP was able to move from cell-to-cell even in absence of functional CP, however systemic movement was abolished.

The CP gene of CMV and CMVcymMP was replaced with the GFP gene in order to visualize the movement of the viruses. The macroscopic and microscopic observations confirmed the results mentioned above. Unlike the original CMV, the hybrid virus (CMVcymMP) was able to move from efficiently from cell to cell without the presence of the CP, but it was not able to move systemically.

CONCLUSIONS

The CMVcymMP is a particularly viable hybrid virus. The reasons for the unusual virulence could be:

(a) high compatibility between the CMV as genetic background and the MP of CymRSV;

(b) the MP of CymRSV is an exceptionally effective movement protein *per se*;

The ability of the hybrid virus to move from cell-to-cell in absence of the CP indicates that a movement mechanism different from the movement of the CMV appears in case of CMVcymMP. This movement mechanism is presumably typical for CymRSV. Since during the course of CMV movement the CP influences the conformation of the viral RNA:MP complex, it is likely that in case of CymRSV MP there is no need for such kind of influence of the CP. Then again, the decreased movement efficiency of the CP deficient hybrid indicates some kind of effect of the CP on the local movement of the hybrid virus.

Different reasons can be found for the lack of systemic movement in case of the CP deficient hybrid virus:

(a) the virus can not transit the phloem-parenchyma boundary (the CP is likely to have a role in this process);

(b) the CMV moves in the phloem in form of virions, and since there are no CP units necessary to build up the virus particles, the long distance movement is not possible;

The size and morphology of local lesions caused by the hybrid virus on *Chenopodium* species were determined by the MP gene of CymRSV. The difference between the lesions caused by the hybrid virus (mostly chlorotic) and by CymRSV (primarily necrotic) rises most likely from the lack of CymRSV protein p19.

The symptoms caused by CMVcymMP on the common systemic hosts of the parental viruses are greatly influenced by the foreign MP gene, however the effect of CMV can be observed as well. As a result, the symptoms of the hybrid virus show a transition between the parental phenotypes.

In case of *N. debneyi* the MP gene is not the factor, that prevents the long distance movement of CymRSV in this plant, since the CMVcymMP containing the MP of CymRSV is able to move systemically in this plant.

Not the MP initiates HR on *N. tabacum* cv. Xanthi, since the CMVcymMP does not cause HR on this plant.

On *N. glutinosa* the MP of CymRSV is responsible for HR induction, since in contrast CMV, the hybrid CMVcymMP carrying the MP of CymRSV induces local lesions on this plant.

PUBLICATIONS

Papers published in peer reviewed scientific journals

Salánki K., Gellért Á., Huppert E., Náray-Szabó G. and Balázs E. 2004. The compatibility of the movement protein and the coat protein of cucumoviruses is required for cell-to-cell movement. *J. Gen. Virol.* 85:1039-1048.

Huppert E., Szilassy D., Salánki K., Divéki Z. and Balázs E. 2002. Heterologous movement protein strongly modifies the infection phenotype of cucumber mosaic virus. *J. Virol.* 76:3554-3557.

Oral presentations, posters

Huppert E., Szilassy D., Salánki K., Divéki Z. and Balázs E. (2002): Hybrid cucumber mosaic virus moves efficiently with the movement protein of cymbidium ringspot virus. XIIth International Congress of Virology, Paris.

Salánki K., Huppert E., Gellért Á. and Balázs E. (2002): The compatibility of the 3a protein and the coat protein of cucumoviruses is required for cell-to-cell movement. XIIth International Congress of Virology, Paris.

Salánki K., Huppert E. és Balázs E. (2001): Cucumovírusok lokális terjedésének feltétele a mozgási fehérje és a köpenyfehérje kompatibilitása. 47. Növényvédelmi Tudományos Napok, Budapest, 2001. február 27-28.

Huppert E., Szilassy D., Salánki K., Divéki Z. és Balázs E. (2001): A mozgási fehérje kicserélése nem okozza az uborka mozaik vírus fertőzőképességének elvesztését. 47. Növényvédelmi Tudományos Napok, Budapest, 2001. február 27-28.

Huppert E., Szilassy D., Salánki K., Divéki Z. és Balázs E. (2001): Hibrid uborka

mozaik vírus előállítása nem homológ mozgási fehérje felhasználásával. Magyar Mikrobiológiai Társaság Nagygyűlése, Balatonfüred, 2001. október 10-12.

Salánki K., Huppert E. és Balázs E. (2000): Cucumovírusok lokális terjedésének feltétele a 3a protein és a köpenyfehérje kölcsönhatása. Magyar Mikrobiológiai Társaság Nagygyűlése,
Keszthely, 2000. augusztus 24.-26.

Huppert, E., Salánki, K., Szilassy, D. és Balázs, E. (1998): Paradicsom magtalanság vírus (TAV-P) egyes és kettes RNS-ének molekuláris jellemzése. 44. Növényvédelmi Tudományos Napok, Budapest, 1998.

REFERENCES

Beczner, L., Devergne, J.C. and Vassányi, R. 1978. Symptomatological and serological characterization of some Hungarian cucumovirus isolates. 3rd International Symposium for Plant Pathology, München, August 16-23 P45.

Burgyán, J., Nagy, P.D. and Russo, M. 1990. Synthesis of infectious RNA from full-length cloned cDNA to RNA of cymbidium ringspot tombusvirus. J. Gen. Virol. 71:1857-60.

Carrington, J.C., Kasschau, K.D., Mahajan, S.K. and Schaad, M.C. 1996. Cell-to-cell and long distance transport of viruses in plants. Plant Cell 8:1669-1681.

Dalmay, T., Rubino, L., Burgyán, J. Kollár, Á. and Russo, M. 1993. Functional analysis of cymbidium ringspot virus genome. Virology 196:697-704.

Edwardson, J.R. and Christie, R.G. 1991. Cucumoviruses. *In* „CRC Handbook of viruses infecting legumes" pp. 293-319- CRC Press, Boca Ratnon, Florida.

Hollings, M., Stone, O. M. and R. J. Barton. 1977. Pathology, soil transmission and characterisation of cymbidium ringspot, a virus from cymbidium orchids and white clover (*Trifolium repens*). Ann. Appl. Biol. 85:233-248.

Hull, R. 2002. Matthew's Plant virology. Academic Press Inc.:San Diego, USA.

Lot, H. and Kaper, J.M. 1976. Further studies on the RNA component distribution among the nucleoproteins of cucumber mosaic virus. Virology 74:223-225.

Palukaitis, P. and García-Arenal, F. 2003. Cucumoviruses. Adv. Vir. Res. 62:241-323.

Salánki, K., Thole, V., Balázs, E. and Burgyán, J. 1994. Complete nucleotide sequence of the RNA3 from subgroup II of cucumber mosaic virus (CMV) strain: Trk7. Virus. Res. 31:379-384.

Salánki, K., Carrére, I., Jacquemond, M., Balázs, E. and Tepfer, M. 1997. Biological properties of pseudorecombinant and recombinant strains created with cucumber mosaic virus and tomato aspermy virus. J. Virol. 71:3597-3602.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Szilassy, D., Salánki, K. and Balázs, E. 1999. Molecular evidence for the existence of two distinct subgroups in cucumber mosaic cucumovirus. Virus Genes 18:221-227.

White, J.L. and Kaper, J.M. 1989 A simple method for detection of viral satellite RNAs in small tissue samples. J. Virol. Methods 23:83-94.