

CROSS-PROTECTION BETWEEN DIFFERENT PATHOTYPES OF *Pepino mosaic virus* REPRESENTING CHILEAN 2 GENOTYPE

Beata Hasiów-Jaroszewska, Julia Minicka, Henryk Pospieszny
Institute of Plant Protection – National Research Institute in Poznań

Abstract. Viral cross-protection in plants is a phenomenon, where a mild virus isolate can protect plants against damage caused by a severe challenge isolate of the same virus. It has been used on a large scale in cases where no resistant plants are available. We examined differences in cross-protection between pathotypes of *Pepino mosaic virus* representing Chilean 2 genotype. The potential of a mild PepMV-P22 isolate to protect tomato against more aggressive challenge isolates causing yellowing and necrotic symptoms was established. The challenge isolates were PepMV-P5-IY (yellowing), PepMV-P19 (necrotic) and PepMV-P22 K67E (artificial necrotic mutant of PepMV-P22 which differ from PepMV-P22 only by a point mutation). Efficient cross-protection was obtained using mild PepMV-P22 against PepMV-P5-IY. After a challenge inoculation with PepMV-P19 or PepMV-P22 K67E symptoms severity were significantly reduced in comparison to non-protected plants; however, necrotic symptoms appeared two months after coinfection. The real-time PCR analysis revealed that the level of accumulation of the necrotic isolate in tomato plants was even 5–7 times higher than that of PepMV-P22.

Key words: cross-protection, PepMV, viral variants

INTRODUCTION

Pepino mosaic virus (PepMV) is currently considered as one of the most dangerous pathogens infecting tomato crops worldwide. PepMV belongs to the *Potexvirus* genus. The RNA genome of PepMV encompasses approximately 6.4 kb and contains five open reading frames that encode RNA-dependent RNA polymerase (RdRp), a triple gene block (TGB) and a coat protein gene (CP). The genome is flanked by two short untranslated regions (UTRs) in the 5' and 3' ends [Maroon-Lango et al. 2005, Ling 2007, Hasiów et al. 2008]. Based on phylogenetic analysis four different genotypes have been described so far: European (EU), Peruvian (LP), the American (US1) and Chilean 2

Corresponding author: Beata Hasiów-Jaroszewska, Department of Virology and Bacteriology, Institute of Plant Protection – National Research Institute, Wł. Węgorka 20, 60-318 Poznań, Poland, e-mail: b.hasiow@iorpib.poznan.pl

(CH2) [Hanssen et al. 2009]. The CH2 genotype is the most widespread and dominant especially in Europe. Isolates belonging to the CH2 genotype share a very high nucleotide sequence similarity ranging from 98 to 100%. It has been shown that single nucleotide substitutions play a role in the development of symptoms on tomato plants. The K67E substitution in TGB3 of a necrotic isolate affected the development of necrosis symptoms on tomato plants [Hasiów-Jaroszewska et al. 2009a, 2011a]. It has also been shown that two separate point mutations (E155K and D166G) in coat protein of other isolates identified in several countries resulted in the development of yellowing symptoms on tomato plants. Moreover, the yellowing isolates induce necrosis on tomato fruits and affected on crops yield and quality [Pospieszny et al. 2011, Hasiów-Jaroszewska et al. 2013]. Amino acids identified in TGB3 and CP are located on the surface of proteins and might play a role in protein-protein interactions during viral infection [Hasiów-Jaroszewska et al. 2011b, 2013].

Up to date, sources of resistance have been identified in the case of wild tomatoes (*Solanum peruvianum*) [Ling and Scott 2007, Soler-Aleixandre et al. 2007], however no resistant cultivars against PepMV are commercially available. For this reason, cross-protection may offer an alternative strategy to reduce economic losses. Cross-protection is a phenomenon where a mild virus isolate can protect plants against damage caused by a severe challenge isolate of the same virus [Ziebell and Carr 2010, Zhou and Zhou 2012]. Cross-protection has been applied to control various viral diseases: *Tobacco mosaic virus* [Rast 1972] and *Zucchini yellow mosaic virus* [Lecoq and Lemaire 1991]. A mild isolate of *Papaya ringspot virus* (PRSV) was used in papaya fields in Hawaii, Taiwan and Mexico [Gal-On and Sibolet 2006]. On the other hand, application of cross-protection may result in the emergence of virus variants with new traits resulting from recombination between protected and challenge isolates.

The aim of this study was to check the potential of the Polish mild isolate of PepMV to protect plants against two others pathotypes, namely yellowing and necrotic. We also estimated replicative fitness of particular isolates in single infection. In this paper, we described results of cross-protection experiments between genetically close variants of PepMV from CH2 genotype. We also discussed the potential of cross-protection to contribute the maintenance of crop health in the face of appearance of new viral variants and threats to agricultural production.

MATERIALS AND METHODS

PepMV isolates. PepMV isolates were selected on the basis of their known virus-associated symptoms in tomato. As a protective isolate PepMV-P22 (mild, asymptomatic) [Hasiów-Jaroszewska et al. 2009a] was selected. The challenge isolates were PepMV-P19 (necrotic) [Hasiów-Jaroszewska et al. 2009a], PepMV-K67E (artificial necrotic mutant of PepMV-P22) and PepMV-P5-IY (yellowing) [Hasiów-Jaroszewska et al. 2013]. Full-length infectious clones of PepMV-P22, PepMV-P19, PepMV-P22 K67E and PepMV-P5-IY, representing mild, necrotic and yellowing isolates (fig. 1 A, B, C), respectively, were used to produce viral RNA, according to the previously described procedure [Hasiów-Jaroszewska et al. 2009b]. Transcription was performed

using 1 µg of linear plasmids as a template and the mMessage mMachine T7 according to the manufacturer's protocol (Applied Biosystems, Austin, USA). The concentration of RNA was measured using NanoDrop (ThermoScientific, New Hampshire, USA). RNA obtained after transcription was mechanically inoculated onto *Solanum lycopersicum* plants of the cultivar Beta Lux.

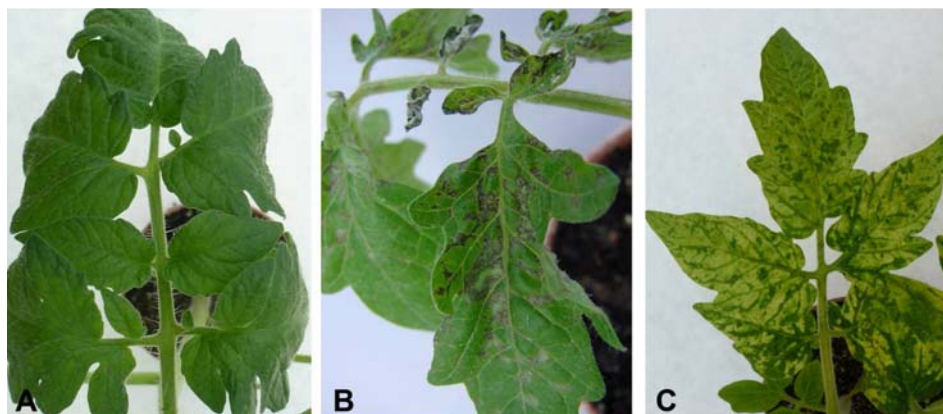


Fig. 1. Tomato plants infected with PepMV-P22, PepMV-P19, PepMV-P5-IY isolates, respectively

Cross-protection experiments. The trials encompassed eight treatments namely the virus-free control treatment, four control treatments of each isolate in a single infection, and three cross-protection treatments. Tomato plants in stage of three leaves were inoculated with the PepMV-P22 mild isolate, known to cause few or no symptoms, subsequently challenged with an aggressive isolate known to cause significant symptoms and damages. Each treatment was performed in three replicates with three plants per replication. RNA obtained from PepMV-P22 was used as protector isolate in the three cross-protection treatments. All rub-inoculation was performed with 40 µl of RNA transcripts (approx. 6–8 µg of RNA) after dusting leaf surfaces with carborundum powder. To determine whether inoculation with all the isolates was successful, plants were sampled 7 days post inoculation (dpi) and examined by the RT-PCR protocol described by Ling et al. [2007]. Two weeks later, plants of the three cross-protection treatments were challenged with PepMV-P5-IY, PepMV-P19 and PepMV-P22 K67E. Mock-inoculation was performed by rubbing plants with phosphate buffer. Plants were monitored for development of symptoms for up to two months. Each treatment was performed in a separate cabin in the greenhouse to avoid contaminating infection between them. The symptoms on cross-protected treatments were compared with symptoms of plants infected with the wild types.

Presence of PepMV in infected plants. The presence of challenge isolates (PepMV-P19, PepMV-P22 K67E and PepMV-P5-IY) in the cross-protection treatments was confirmed two weeks after the second round of inoculation. In addition, the ratio of wild-type (PepMV-P22) versus yellowing (PepMV-P5-IY) and necrotic variants

(PepMV-P19 and PepMV-P22 K67E) was also established two months after challenge infection. Total RNA was isolated from apical parts of the plants using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and subjected to RT-PCR using TGB3F and TGB3R primers [Hasiów-Jaroszewska and Borodynko 2013] and CPD and CPR primers [Pagán et al. 2006]. The TGB3F/R amplified a 500-bp product encompassing the entire TGB3 region, whereas CPD/R amplified a 842-bp product including complete CP region of PepMV. RT-PCR amplification was performed using 1 µl of RNA (1 µg·µl), primers for CP and TGB3 at a final concentration of 400 nM and Transcriptor One-Step RT-PCR Kit (Roche, Mannheim, Germany), according to the manufacturer's protocol. The reaction was run in a Thermal Cycler (Biometra GmbH, Göttingen, Germany) with the following program: 30 min at 50°C for reverse transcription, 1 cycle of 7 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 47°C and 51°C for CP and TGB3, respectively, 30 s at 68°C and finally 1 cycle of 68°C for 5 min. After amplification RT-PCR products were separated and verified on a 1% agarose gel. Obtained products of the appropriate size were purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) according to manufacturer's protocol, then ligated into pGEM-Teasy and transformed into *E. coli* competent cells (Invitrogen, Grand Island, USA). Obtained plasmids were isolated using the Inorb Spin Plasmid Mini Two kit (STRATEC molecular, Berlin, Germany) and cut with the EcoRI restriction enzyme to verify cloning efficiency. Ten recombinant plasmids were sequenced, in each case using universal primers hybridizing to the vectors M13F and M13R. The sequences were specifically verified for the presence of mutations in TGB3 (K67E) and CP (E155K) indicating the presence of challenge isolates in plants.

RNA quantification of PepMV isolates. Fitness of mild, yellowing and necrotic isolates of PepMV in single infection in tomato (cv. Beta Lux) was estimated. The relative quantity of viral RNA was measured by real time quantitative PCR (RT-qPCR) using LightCycler® 96 (Roche). A set of 15 tomato plants was mechanically inoculated with RNA transcribed from the infected clones (PepMV-P22, PepMV-P5-IY, PepMV-P19, PepMV-P22 K67E) as described above. Mock-inoculated plants served as controls. Rub-inoculation was performed with 40 µl of RNA transcripts (approx. 6–8 µg of RNA) after dusting leaf surfaces with carborundum powder. Total RNA from all the infected plants was isolated at 7, 14 and 21 dpi using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA concentration was measured at least twice with a NanoDrop® ND-1000 spectrophotometer for each preparation and then diluted to 100 ng·µl using total RNA extract (10 ng·µl) from healthy tomatoes as diluents. RNA was reverse transcribed using the High Fidelity Transcriptor cDNA (Roche) and the oligodT primer at a final concentration of 2.5 µM, according to the manufacturer's protocol. For the preparation of the standard curve 10-fold serial dilutions (from 1 µg·µl to 10 pg·µl) of the cDNA were prepared. The obtained cDNA was used in real-time PCR using FastStart DNA Master SYBR Green I (Roche) and PepVF1/PepVR1 primers [Hasiów-Jaroszewska and Komorowska 2013], according to manufacturer's protocol. The slope values were estimated plotting the threshold cycle (*C_t*) values from two independent assays with three replicates each. Relative quantity of viral RNA in each sample was estimated by interpolating individual *C_t* values in the standard curve from two independent RT-qPCR assays, using the LightCycler® 96 SW 1.1 software (Roche).

RESULTS

PepMV presence. The presence of PepMV in treatments was confirmed by RT-PCR. Mock- inoculated plants were virus-free. Cloning and sequencing of the TGB3 and CP regions confirmed the establishment of protector and challenge isolates in the cross-protection treatments. Interestingly, after two months of infection with challenge isolates in plants co-inoculated with PepMV-P5-IY only the CP sequences typical for PepMV-P22 were observed. In plants inoculated with necrotic variants the ratio of clones bearing wild type variants of TGB3 versus the necrotic variants was 4:6.



Fig. 2. Cross-protected tomato plants: a – infected with PepMV-P22 and then challenged by PepMV-P5-IY, b – infected with PepMV-P22 and then challenged by PepMV-P19. Both photos were taken two months after infection

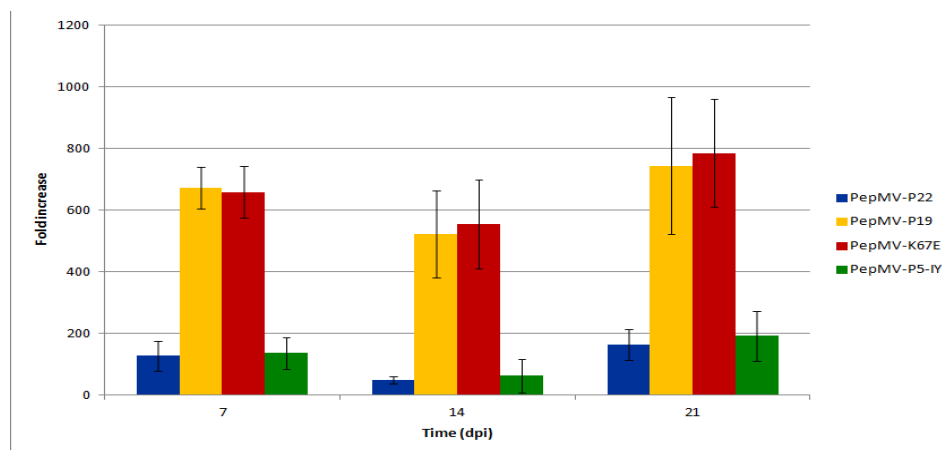


Fig. 3. Fold changes in accumulation of different isolates of *Pepino mosaic virus*: PepMV-P22, PepMV-P5-IY, PepMV-P19 and PepMV-P22 K67E at 7, 14 and 21 dpi. Relative quantity of viral RNA was calculated using LightCycler® 96 SW 1.1 software (Roche). Bars indicate standard error of the mean

Cross-protection experiments. Symptoms typical for each isolate were observed on plants two weeks after single isolate inoculation. The challenging isolates induced significantly more severe symptoms than PepMV-P22. PepMV-P5-IY infected plants started to show yellowing symptoms, which covered whole plants after two weeks. The symptoms induced by PepMV-P5-IY were not observed in the cross-protected plants (fig. 2A). PepMV-P19 and PepMV-P22 K67E were the most aggressive and induced necrotic spots on control plants after two weeks. Plants infected with the challenge isolates of PepMV-P19 and PepMV-P22 K67E did not show necrotic symptoms until two months of co-inoculation (fig. 2B). Then, symptoms appeared and even though less severe, they never disappeared entirely.

RNA quantification of PepMV isolates. Relative amounts of viral RNA of PepMV-P22, PepMV-P5-IY, PepMV-P19 and PepMV-P22 K67E were measured by RT-qPCR in sets on inoculated tomato plants after 7, 14 and 21 dpi. Virus accumulation differed significantly, depending on the isolate and type of infection. On average PepMV-P19 and PepMV-P22 K67E accumulation was 5–7 times greater in comparison to PepMV-P22 (fig. 3). In the case of the yellowing isolate (PepMV-P5-IY) the accumulation rate was quite similar to that of PepMV-P22 in tomato plants at all measurement days (fig. 3). No amplification plots were obtained for mock-inoculated plants.

DISCUSSION

Cross-protection is suggested to perform well when the genetic relationship between protecting and challenging variants is high. In our experiments we used phylogenetically related isolates which shared 98.7–99.9% of overall sequence identity. In this study we examined the potential of a mild CH2 isolate (PepMV-P22) to provide cross-protection in tomato crops against more severe isolates from CH2 genotype which cause yellowing and necrotic symptoms. The PepMV-P22 isolate reduced the effects of PepMV isolates with aggressive symptoms effectively. After challenge inoculation, symptom severity was significantly reduced in comparison to the non-protected plants. The best results were obtained with PepMV-P5-IY isolate, where protected plants did not display any of yellowing symptoms after challenge inoculation. It has been previously shown that yellowing mutations are unstable and tend to be back-mutated. Experiments with wild-type and mutated infectious clones showed that back-mutation towards the wild-type sequence, rather than a difference in accumulation speed or efficiency, is responsible for the disappearance of the yellowing symptoms [Hasiów-Jaroszewska et al. 2013].

After challenge inoculation with PepMV-P19 and PepMV-P22 K67E symptom severity was significantly reduced in comparison to non-protected plants; however, necrotic symptoms appeared after two months of co-infection. Although PepMV-P19 and PepMV-P22 share 99% identity and belong to the same genotype, PepMV-P22 did not induce a persistent cross-protection against necrotic CH2 isolate. Moreover, the plants infected with challenge isolates of the PepMV-P22 K67E mutant, which differs only in single nucleotide substitution from PepMV-P22, displayed necrosis symptoms after two months of infection. This indicated that RNA sequence homology is not the only factor

affecting the efficiency of cross-protection methods. The 99.9% sequence identity was not sufficient to provide persistent protection of tomato crops. It has been shown that the codon 67 of TGB3 is under positive selection pressure and potentially variants bearing this mutation have evolutionary advantages over the mild CH2 genotype [Hasiów-Jaroszewska et al. 2011b]. Our results clearly indicate that accumulation of necrotic variants in comparison to mild ones was significantly higher. It suggests that the decision to apply cross-protection vaccination should be preceded with trials involving different types of isolates, especially taking into account the choice of protector isolate. Practical cross-protection requires mild or attenuated isolate of the virus which is genetically stable and its level of accumulation is sufficient to protect plants against more aggressive variants. A study performed by Hanssen et al. [2010] revealed that efficient cross-protection against the prevalent CH2 genotype of PepMV can be obtained by pre-inoculation with a mild CH2 isolate, but enhanced symptom severity can occur when the protector and challenge isolates belong to different genotypes (EU, LP). This can be the result of synergism between different PepMV genotypes or recombinants arising during the co-infection [Hanssen et al. 2010].

Although several hypotheses have been proposed to explain the molecular mechanism underlying cross-protection, no single hypothesis can account for all the data obtained. Several cross-protection models have been proposed: 1) transcription of the incoming viral nucleic acid may be prevented even if it is initially translated, 2) the production of genome length RNA could be inhibited even if the challenge virus is replicated, 3) cell-to-cell movement could be prevented [Beachy 1999, Sherwood 1987], and 4) pre-activation of the RNA-induced silencing complex (RISC) with small interfering RNA (siRNA) derived from the protector virus RNA, thus inhibiting replication of the challenge isolate [Ratcliff et al. 1999, Gal-On and Siboleth 2006]. Apart from the above mentioned mechanisms, a management strategy based on cross-protection can only be successful in areas where one PepMV genotype is dominant, provided that the PepMV population is monitored intensively and that very strict hygiene measures are taken during cultivation and between different cropping cycles [Hanssen et al. 2010].

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OCHRONA KRZYŻOWA POMIĘDZY RÓŻNYMI PATOTYPAMI WIRUSA MOZAIKI PEPINO REPREZENTUJĄCYMI GENOTYP CHILIJSKI 2

Streszczenie. Zjawisko ochrony krzyżowej (ang. cross-protection) zachodzi tylko pomiędzy izolatami pokrewnymi danego gatunku wirusa. Polega ono na celowym zakażeniu roślin bardzo łagodnym izolatem wirusa (izolat ochronny), aby chronić je przed innym, ostrym izolatem tego samego wirusa (izolat konkurencyjny). W prezentowanej pracy analizowano potencjał wykorzystania łagodnego izolatu (PepMV-P22) wirusa mozaiki pepino (*Pepino mosaic virus*, PepMV) do ochrony roślin pomidora przeciwko innym izolatom, powodującym zróżnicowane objawy na roślinach. Jako izolaty konkurencyjne wykorzystano: PepMV-P5-IY (żółtaczkowy), PepMV-P19 (nekrotyczny) oraz PepMV-P22 K67E (mutant nekrotyczny, różniący się jedynie pojedynczą mutacją od PepMV-P22). Zjawisko ochrony krzyżowej zachodziło efektywnie w przypadku wykorzystania PepMV-P22 przeciwko PepMV-P5-IY. W przypadku PepMV-P19 oraz PepMV-P22 K67E ochrona krzyżowa została przełamana, jednakże symptomy były mniej intensywne i pojawiły się później niż u roślin, u których nie stosowano ochrony krzyżowej. Ponadto analiza real-time PCR wykazała, że akumulacja wirusa w przypadku nekrotycznych wariantów była około 5–7 razy większa w porównaniu z łagodnym izolatem wirusa.

Słowa kluczowe: ochrona krzyżowa, PepMV, warianty wirusa

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