

PEPINO MOSAIC VIRUS – A PATHOGEN OF TOMATO CROPS IN POLAND: BIOLOGY, EVOLUTION AND DIAGNOSTICS

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Abstract: *Pepino mosaic virus* (PepMV) has the potential to cause serious tomato disease. Several isolates of PepMV were collected between 2003–2010 in the Department of Virology and Bacteriology, Institute of Plant Protection – National Research Institute, Poznań, Poland. The isolates were obtained from greenhouse tomato plants (*Solanum lycopersicum*) collected from different regions of Poland. Clear biological and genetic differences were detected among the Polish PepMV isolates. Since the latter induced a wide range of symptoms on tested plant species, five different pathotypes were distinguished. Sequence analysis showed that the Polish PepMV isolates share a very high sequence identity with those representing European and Chilean 2 genotype. The analysis of the genetic diversity of the Polish PepMV CH2 isolates suggested that each of them exists as a genetically diverse collection of variants called a quasispecies.

Key words: PepMV, genetic diversity, host range, symptoms, diagnostic

Pepino mosaic virus (PepMV) was first described in 1980 after it was found to infect pepino (*Solanum muricatum*) in Peru (Jones *et al.* 1980). In 1999, the virus was detected in tomato (*S. lycopersicum*) in the Netherlands and the United Kingdom (Wright and Mumford 1999; van der Vlugt *et al.* 2000). Subsequently, outbreaks of the disease were reported in many other European countries as well as in the United States and Chile (Cotillon *et al.* 2002; French *et al.* 2001; Hanssen *et al.* 2008; Hasiów *et al.* 2008a; Mumford and Metcalfe 2001; Pagan *et al.* 2006; Pospieszny *et al.* 2003; van der Vlugt *et al.* 2000; Ling 2007; Maroon-Lango *et al.* 2005). Comparative study of European and non-European PepMV isolates revealed striking differences in symptoms they induced and in their host range. PepMV infection can be accompanied by mild mosaic, leaf chlorosis, bubbling of the leaf surface as well as plant necrosis. Sequence analysis also revealed a high level of genetic diversity among the isolates and within them. So far, four major PepMV genotypes (with an intergenotype RNA sequence identity ranging from 78 to 95%) have been distinguished: the original Peruvian (LP); European (EU); American 1 (US1) and Chilean 2 (CH2). However, phylogenetic classification of sequences is usually confounded by the presence of recombinant or mosaic genomes wherein the distinct sequence regions do not have a common evolutionary descent (Awadalla

2003). Therefore, recombination must be taken into account when drawing conclusions about the evolutionary origins of PepMV isolates. RNA recombination has been reported for Belgian PepMV isolates co-infecting tomato plants (Hanssen *et al.* 2008). In this study, two PepMV genotypes; EU and CH2, were found to recombine in mixed infections under natural conditions. In addition, other PepMV genotypes including LP, EU and US1 have also been found to co-infect tomato plants (Pagán *et al.* 2006), raising concerns about crop management in the presence of recombinant PepMV variants.

In Poland, several isolates belonging to EU and CH2 genotypes have been found since 2003 (Pospieszny *et al.* 2003; Pospieszny and Borodynko 2006; Pospieszny *et al.* 2008). Two different isolates denoted PepMV-SW and PepMV-No representing EU genotype were found in 2002 and 2007, respectively. In 2005–2010 a collection of isolates of the CH2 genotype were established. Among them, four isolates which caused severe necrosis on tomato were found (PepMV-Pa, PepMV-Ku, PepMV-Ros, PepMV-Fr) (Hasiów-Jaroszewska *et al.* 2009b). Another isolate, which was found in 2010, induced severe chlorosis on tomato plants leading to yield and quality losses. The isolates differed in their host range, and symptoms. They also differed genetically.

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In this paper we review what is currently known about the Polish PepMV isolates: their biology, genome diversity, population dynamics, detection and transmission.

Host range and symptomatology

PepMV symptomatology and host range have been extensively studied during the last decade. General symptoms in tomato included mosaic and yellowing of leaves, bubbling, necrosis, and alteration of the fruit color resulting in uneven ripening. Last spring and summer (2010), all Polish isolates of both genotypes (EU and CH2) did not bring about any remarkable symptoms or only very mild mosaic and fruit marbling. Symptoms seemed to be related to environmental conditions and possibly the cultivar. Low temperature and low light conditions are thought to result in more severe damage (Jorda *et al.* 2001). Based on symptoms induced and host range we distinguished five different pathotypes among the Polish isolates (Table 1). Two of them, severe PepMV-SW and mild PepMV-No, belong to the EU genotype. Severe pathotype, in comparison to a mild one, had a wider host range and caused more severe symptoms. PepMV-SW infected most species from *Nicotiana* genus and *Physalis floridana*. *P. floridana* was not infected by other Polish isolates. Furthermore, the host range of PepMV-SW included potato (*Solanum tuberosum*) and pepper (*Capsicum annuum*). Taking into account the host range and symptoms, PepMV-SW was more similar to the Peruvian genotype than to the European. Pepino isolate from LP genotype (denoted BBA1137) alone caused symptoms in *Nicotiana tabacum*, *C. annuum* and *P. floridana* (Verhoeven *et al.* 2003). Pathotype PepMV-No had limited host range and caused rather mild symptoms which were similar to those induced by mild isolates from CH2 genotype. The three next pathotypes: necrotic PepMV-Pa, mild PepMV-PK and yellowing (PepMV-ZI) belong to the CH2 genotype. The necrotic isolates caused only mild symptoms during spring and summer, whereas at lower temperature they caused necrosis on tomato, and as a consequence the plants died (Fig. 1). Another characteristic fea-

ture of necrotic isolates was the induction of local necrotic spots on *Datura innoxia*. A different isolate representing the CH2 genotype induced severe yellowing and chlorosis on tomato plants (Fig. 2). Mild CH2 isolates did not induce any significant symptoms. The necrotic isolates shared over 99% of sequence identity with the mild isolates from the CH2 genotype. This suggests that minor differences in genome sequence can cause significant differences in symptomatology between isolates that infect crops under the same conditions. However, in a population study conducted on Spanish tomato crops, no correlation between PepMV genotypes and symptoms was found (Pagan *et al.* 2006). The evidence of variability may indicate that the virus has an ability to withstand environmental fluctuations, to adapt to a wider range of habitats or hosts, to develop resistance to plant protection products and to overcome host resistance. Accordingly, cross-protection of plant viruses is a phenomenon in which plants infected with one strain of a virus are protected from the effects of superinfection with other related strains. Practical use of cross-protection has been reported for the control of plant viruses in economically important crops: *Tomato mosaic virus* (ToMV) in tomato (Rast 1972; Oshima 1975; Fletcher 1978), *Papaya ringspot virus*-type P in papaya (Wang *et al.* 1987) and *Cassava mosaic viruses* in cassava (Owor *et al.* 2004). The potential of mild PepMV-PK isolate belonging to the CH2 genotype to protect a tomato crop against a challenge with an aggressive isolate of the CH2 genotype (PepMV-Pa) was assessed in greenhouse trials. PepMV symptoms were rated at regular time points (Pospieszny 2007a). After a challenge infection, enhanced symptom display was recorded in plants that were pre-inoculated with a protector isolate from the EU genotype. By contrast, efficient cross-protection was obtained using the mild isolate of the CH2 genotype. In this case the challenge isolate was barely detectable in the pre-inoculated plants (Hanssen and Thomma 2010; Pospieszny 2007a). These results confirm that interaction between PepMV isolates depends on RNA sequence homology.

Table 1. Host range and symptoms induced by different Polish PepMV isolates

| Plants | Symptoms | | | | |
|------------------------------------|-------------|----------|--------------|------------|----------|
| | EU genotype | | CH2 genotype | | |
| | PepMV-No | PepMV-SW | PepMV-PK | PepMV-Pa | PepMV-ZI |
| <i>Ch. quinoa</i> | – | L, – | L, – | – | – |
| <i>C. sativus</i> | – | – | – | – | – |
| <i>N. tabacum</i> cv. Xanthi | (s) | (L), S | (s), (S) | – | – |
| <i>N. tabacum</i> cv. Samsun | (s) | –, S | (s) | – | – |
| <i>N. tabacum</i> cv. White Burley | (s) | L, S | s, S | – | – |
| <i>N. benthamiana</i> | L, S | L, S | L, S | L, S | L, S |
| <i>N. glutinosa</i> | L, S | L, S | s, S | s, S | s, S |
| <i>N. clevelandii</i> | L, S | L, S | L, S | L, S | L, S |
| <i>S. lycopersicum</i> | –, S, s | –, S, s | –, s, S | Ln, s, Sn, | Lch, SYL |
| <i>D. innoxia</i> | (L), S | L, S | L, S | Ln, S | Lch, S |
| <i>C. annuum</i> | – | s, S | – | – | – |
| <i>P. hybrida</i> | – | – | – | – | – |
| <i>P. floridana</i> | – | L, S | – | – | – |

L – local symptoms; S – systemic symptoms; s – symptomless; – no symptoms, no virus; () occasional infection; Ln – local leaves necrosis; Lch – local chlorosis; Sn – systemic leaf necrosis; SYL – systemic yellowing



Fig. 1. Tomato plant infected by PepMV-Pa



Fig. 2. Tomato plant infected by PepMV-ZL

Genome organization and diversity

PepMV belongs to the *Potexvirus* genus of the *Flexiviridae* family. Virions are non-enveloped flexuous rods of 500 nm in length (Jones *et al.* 1980). PepMV possesses a single, positive sense genome (+ssRNA), ca. 6 400 nt in length. The genomic RNA contains five open reading frames (ORFs) and two small inter-cistronic regions. ORF1 encodes for the putative viral polymerase (RdRp) (Aguilar *et al.* 2002). ORFs 2, 3 and 4 encode the triple gene block (TGB) proteins: TGBp1, TGBp2 and TGBp3, which are essential for virus movement (Morozov and Solovyev 2003; López *et al.* 2005) and ORF5 encodes the coat protein gene (CP). Finally, two short untranslated sequences flank the coding regions and there is a poly(A) tail at the 3' end of the genomic RNA (Cotillon *et al.* 2002). The full length genomic sequences of PepMV-PK and PepMV-Pa were determined (Hasiów *et al.* 2008a; Hasiów-Jaroszewska *et al.* 2009a). Analysis showed an almost 99% sequence identity between them. Analysis also revealed the highest sequence similarity with PepMV-Ch2 isolate originating from tomato seed produced in Chile. So far, several sequences of isolates representing CH2 genotype have been deposited in the GenBank database and all of them are very similar. Polish CH2 isolates shared a 78–80% identity with LP and EU isolates and a 78% identity with US1 and Ch1 isolates from the USA and Chile, respectively. The partial sequence of PepMV-SW and PepMV-No isolates revealed a 99% sequence similarity between them. Phylogenetic analysis showed that all PepMV isolates representing the EU genotype clustered together on a phylogenetic tree. They shared a 96–97% sequence identity with the Peruvian isolates. Comparative analyses based on five genomic regions (RdRp, TGB1, TGB2, TGB3 and CP) revealed that phylogenetic relationships between the isolates depends on the genome fragment used in analysis and the type of the analyzed sequence (amino acid or nucleotide one). The isolates classified as EU strains, grouped together irrespective of the region analyzed. The homology between isolates suggested their common origin. More differences between isolates belonging to the CH2 strain were observed. The variations in their genomes were not evenly distributed; instead they were concentrated in the region spanning the second half of replicase and the first half of the TGB1 gene. Afterwards, phylogenetic analysis revealed three main clusters, the first containing EU and LP genotypes, the second consisting of US1 and Ch1 isolates and the third containing the CH2 genotype. Minor nucleotide differences were observed between necrotic PepMV-Pa and other non-necrotic isolates. Comparison of 12 full length genomes originating from different countries revealed 10 unique point mutations in PepMV-Pa which affect amino acid composition (Hasiów-Jaroszewska *et al.* 2009a). Most of the mutations were located in the RdRp gene.

It has been shown previously that a single amino acid substitution can change the virus properties. Necrotic isolates of *Cucumber mosaic virus* caused necrosis of several *Nicotiana* species, in contrast to other isolates causing only systemic mosaic. The differences in symptomatology are caused by a single change of arginine to cysteine in 1a protein (Diveki *et al.* 2004). The virulence of ZYMV

also depends on one mutation in the P3 gene (arginine to aspartic acid codon) resulting in different symptoms on *Cucurbita pepo* (mild vs. severe) (Desbiez *et al.* 2003).

PepMV genotypes and population dynamics

Knowledge of the epidemiology and evolution of pathogen populations is essential for understanding the emergence of new diseases. Among all PepMV genotypes, CH2 was shown to be the most prevalent, causing more than 90% of the infections. Since 2003, the first finding of PepMV-SW, EU genotype was found once more in 2007 (Pospieszny *et al.* 2003). Remarkably, while the CH2 genotype had not been previously detected in European tomato, we found that this genotype was present in 99% of the surveyed greenhouses with PepMV infected tomato crops in Poland in 2010. The widespread occurrence of the CH2 genotype suggests that it may have biological advantages over the EU genotype in Polish climate conditions. In several European countries, the CH2 genotype has now become dominant and has largely replaced the EU genotype (Davino *et al.* 2008; Hanssen *et al.* 2008). However, it was suggested that in seed transmission the EU genotype has an advantage over the CH2 genotype and it might explain the original dominance of the European genotype in European countries in the initial outbreaks (Hanssen *et al.* 2010). In addition, the EU genotype is still predominant in the USA (Ling 2008). In Spain, although EU genotype was dominant other genotypes and mixed infections also occurred (Pagan *et al.* 2006). Competition with other viruses is not likely to have a limiting effect on PepMV. In fact PepMV has been found in mixed infections with *Tomato chlorosis virus* in Italy (Davino *et al.* 2008), while co-infections with *Tomato yellow leaf curl virus* and *Tomato torrado virus* were reported in Spain (Soler-Aleixandre *et al.* 2005; Alfaro-Fernandez *et al.* 2010). Polish PepMV was less diverse and no interstrain recombinants were detected. Co-infection with *Tomato torrado virus* (ToTV) was observed and interestingly, the co-infection of ToTV and PepMV in Poland was mainly associated with more severe symptoms. The occurrence of new genetic variants indicated a rapid evolution of PepMV. Analysis of single nucleotide polymorphism showed that among different isolates collected from 2005 to 2010, some displayed higher mutation rates than others. Generally, very few nonsynonymous substitutions were observed which suggests a strong purifying selection. The symptoms induced by different non-necrotic PepMV isolates of the CH2 genotype were very similar which suggests that most of the mutations that take place have no clear biological relevance. These results are in line with those published by Gomez (2009) and Hanssen *et al.* (2010). However, the occurrence of a new necrotic type of PepMV isolate of the CH2 genotype indicates that PepMV may create different genetic variants that induce different symptoms. Our results suggest that even a single nucleotide substitution may have a dramatic effect on the virulence of virus isolates and may result in severe phenotypic changes. The factors contributing to PepMV population dynamics remain unknown. Analysis of the genetic diversity of the Polish CH2 isolates suggests that PepMV may consist of a genetically diverse collection of

variants. The dominant members may vary during shifts among successive host varieties, in the manner of what is called a quasispecies (Roosinck 1997; Schneider and Roosinck 2001). An RNA virus population, termed a quasispecies, is not homogenous; rather, it is an ensemble of slightly differing related sequences. Quasispecies arise from rapid genomic evolution powered by a high mutation rate during viral replication. We have attempted a qualitative characterization of the PepMV genomes, on the assumption that it does exist as a collection of sequences varying around its own consensus sequence. Sequence variation among isolates was characterized by the mean Hamming distance (MHD) (Kędziora *et al.* 2005) and phylogenetic trees were created to depict relations between the identified variants. The MHD, being the average of the distances taken for all pairs of the analyzed sequences, reflected the genetic diversity of the population. In the examined populations MHD ranged from 1.02 to 9.58. The sequence data showed a variation from 10 to 15% and from 5 to 9% at the nucleotide and amino acid level, respectively (Hasiów-Jaroszewska *et al.* 2010). This variation implies that different isolates display various levels of genetic polymorphism. Important biological characteristics may be related to the levels of diversity in the quasispecies.

Transmission and diagnostics

PepMV is efficiently transmitted mechanically (Jones *et al.* 1980). The virus is highly contagious in tomato, as it is easily spread by standard crop handling procedures in a glasshouse; through contaminated tools, hands and clothing and by direct plant-to-plant contact (Wright and Mumford 1999; Spence *et al.* 2006). The main pathways of worldwide spread for PepMV is plant material (mainly seedlings and, potentially, infected fruit) as well as contaminated packing material. Some reports have shown that subjecting contaminated seed to growing-out assays did not result in PepMV transmission to the seedlings (Salomone and Roggero 2002; Ling 2008). In other studies, seed transmission has been demonstrated (Krinkels 2001; Córdoba-Selles *et al.* 2007; Hanssen *et al.* 2010). In these studies, rates of transmission were up to 2%, depending on the seed cleaning and disinfection methods applied. Nevertheless, seed transmission represents a high potential risk and should be considered for the development of control strategies based on genetic resistance.

For routine detection of PepMV in plant material, the standard ELISA test or IC-RT-PCR were used (Pospieszny *et al.* 2007b). The serological studies showed that Polish isolates reacted with both antiserum against PepMV-SW and PepMV-PK (Pospieszny *et al.* 2007a). Diagnosis of PepMV based on disease symptoms is not reliable because not all PepMV-infected plants show symptoms. Generally, biological indexing on *Nicotiana benthamiana* is time-consuming and takes 3–4 weeks, while serological diagnosis may not provide the sensitivity that is needed to detect low-level contamination in a large seed lot (Salomone and Roggero 2002). Despite the high level of sequence diversity between PepMV isolates, we were able to determine a conserved region for primer and probe design. Consequently, the new primer set and RT real-

time PCR method were developed for rapid and sensitive detection of PepMV (Hasiów *et al.* 2008b). We also applied high melting resolution (HMR) analyses. This probe-based technique is sensitive enough to detect single-nucleotide polymorphism (SNP) and can distinguish between mutant alleles by virtue of the dissociation patterns produced. HRM analysis is a powerful technique for the detection of mutations, polymorphisms and epigenetic differences in double stranded DNA samples (Reed *et al.* 2007). In our assay, we were able to distinguish different PepMV genotypes by analysis of probe-target melting curves (Hasiów *et al.* 2008b). A more practical application of real-time PCR for PepMV detection of high throughput samples was made possible with immunocapture sample preparation. This approach allowed us to process 96 samples at once in a general ELISA extraction buffer without the time-consuming RNA extraction procedure. We also adapted one step immunocapture RT real-time PCR TaqMan assay, described previously by Ling (2007), for a broad spectrum detection of PepMV isolates. We were able to detect all four PepMV genotypes in a one step reaction. The level of sensitivity obtained in IC-RT real-time PCR allows us to use this technique for routine seed health assays.

In summary, the PepMV is a very dangerous pathogen of tomato due to its rapid evolution and worldwide distribution. The knowledge about PepMV diversity and epidemiology is essential to develop new diagnostics tools and efficient control strategies.

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POLISH SUMMARY

WIRUS MOZAIKI PEPINO – BIOLOGIA, EWOLUCJA I DIAGNOSTYKA

W latach 2003–2010, w Zakładzie Wirusologii i Bakteriologii Instytutu Ochrony Roślin – Państwowego Instytutu Badawczego w Poznaniu, pozyskano kolekcję izolatów wirusa mozaiki pepino (*Pepino mosaic virus*), jednego z najgroźniejszych wirusów infekujących pomidora. Zebrane izolaty wykazywały zróżnicowanie zarówno w zakresie roślin żywicielskich, jak i objawach wywołanych na porażanych roślinach. Na tej podstawie wyróżniono pięć patotypów wirusa. Porównanie sekwencji wybranych izolatów do sekwencji innych izolatów zdeponowanych w Banku Genów, pozwoliło zakwalifikować polskie izolaty do dwóch genotypów: europejskiego (EU) i chilijskiego 2 (CH2). Analiza sekwencji wybranych regionów genomu izolatów z grupy CH2 wykazała, że PepMV tworzy populację zwaną quasi-gatunkiem. Otrzymane wyniki potwierdzają wysoki stopień zróżnicowania zarówno genetycznego, jak i biologicznego pomiędzy polskimi izolatami PepMV.