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Review

Rapid evolutionary dynamics of the *Pepino mosaic virus* – status and future perspectives

Julia Minicka[1]*, *Beata Hasiów-Jaroszewska*[1], *Natasza Borodynko-Filas*[1], *Henryk Pospieszny*[1], *Inge Maria Hanssen*[2]

[1] Department of Virology and Bacteriology, Institute of Plant Protection – National Research Institute, Władysława Węgorka 20, 60-318 Poznań, Poland

[2] De Ceuster Meststoffen Corporation, Bannerlaan 79, 2280 Grobbendonk, Belgium

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Abstract: *Pepino mosaic virus* (PepMV) has emerged as an important pathogen of greenhouse tomato crops and is currently distributed worldwide. Population genetic studies have revealed a shift in the dominant PepMV genotype from European (EU) to Chilean 2 (CH2) in North America and several European countries. New genetic variants are constantly being created by mutation and recombination events. Single nucleotide substitutions in different parts of the genome were found to affect on development of symptoms resulting in new pathotypes and accumulation of viral RNA. The variability of the PepMV population has a great impact on designing specific diagnostic tools and developing efficient and durable strategies of disease control. In this paper we review the current knowledge about the PepMV population, the evolutionary dynamics of this highly infective virus, methods for its detection and plant protection strategies.

Key words: diagnostic methods, evolution, genetic diversity, *Pepino mosaic virus*

Introduction

Pepino mosaic virus (PepMV) belongs to the genus *Potexvirus* in the *Alphaflexiviridae* family. It was first described on *Solanum muricatum* Aiton in Peru in 1974 (Jones 1980). Within a few years (1999–2010), the virus spread throughout the main tomato-growing areas in Europe and North and South America, causing significant losses in quality and yield in tomato production (Wright and Mumford 1999; van der Vlugt *et al*. 2000; French *et al*. 2001; Mumford and Metcalfe 2001; Roggero *et al*. 2001; Cotillon *et al*. 2002; Pospieszny *et al*. 2003; Maroon-Lango *et al*. 2005; Pagan *et al*. 2006; Ling 2007; Hanssen *et al*. 2008; Hasiów *et al*. 2008). Nowadays, PepMV is considered to be one of the most important viruses in greenhouse tomato culture and has been placed on the European Plant Protection Organization alert list (EPPO 2009).

PepMV is a positive-sense single stranded RNA virus of approximately 6.4 kb. Genomic RNA is capped at the 5′ end and polyadenylated at the 3′ end and contains five open reading frames (ORFs1–5), flanked by 5′ and 3′ untranslated regions (UTRs) (Aguilar *et al*. 2002). ORF1 encodes a RNA-dependent RNA polymerase (RdRp) of 164 kDa, containing methyl transferase, NTP-binding and polymerase domains. ORFs2–4 encode the overlapping movement proteins (triple gene block – TGBp1–3) of 26, 14 and 9 kDa, respectively. These movement proteins are involved in viral movement, suppression of RNA silencing and the development of symptoms (Morozov and Solovyev 2003; Hasiów-Jaroszewska *et al*. 2011, Sempere *et al*. 2016). The last ORF encodes a coat protein (CP) of 25 kDa which plays a role in the development of symptoms, encapsidation and virus movement (Maroon-Lango *et al*. 2005; Ling 2007; Hasiów *et al*. 2008). Particles are non-enveloped flexuous rods about 500 nm in length (Jones *et al*. 1980).

PepMV mainly infects plants of the Solanaceae family, such as *Solanum lycopersicum* L., *S. melongena* L. (eggplant), *Datura inoxia* Mill., *D. stramonium* L., *Nicotiana benthamiana* Domin, *Physalis floridana* and *S. tuberosum* L. (potato) (Blystad *et al*. 2015). The symptoms on tomato plants are very diverse (van der Vlugt *et al*. 2000; Hanssen *et al*. 2009) and range from mild or even asymptomatic to very strong, sometimes even resulting in plant death. They may occur in the form of fruit discoloration, marbling, open fruit, leaf blistering and bubbling, leaf chlorosis, leaf yellowing or leaf and stem necrosis (van der Vlugt *et al*. 2000; Roggero *et al*. 2001; Spence *at al*. 2006; Hasiów *et al*. 2008; Hasiów-Jaroszewska *et al*. 2009, 2013; Hanssen and Thomma 2010). The severity of the symptoms on infected tomato plants depends largely on the genotype (Hasiów-Jaroszewska *et al*. 2010a), if it is a single or mixed infection (Hanssen *et al*. 2008; Hanssen *et al*. 2009) and climate

*Corresponding address:
J.Minicka@iorpib.poznan.pl

conditions (Spence *et al.* 2006). The virus is efficiently transmitted mechanically, but very low rates of transmission by seeds, whiteflies, bumble bees and the soil-borne fungus *Olpidium virulentus* have been reported (Córdoba-Sellés *et al.* 2007; Shipp *et al.* 2008; Alfaro-Fernández *et al.* 2009; Hanssen *et al.* 2010a; Noël *et al.* 2014). Water has also been reported as a possible source of PepMV infection (Schwarz *et al.* 2010; Mehle *et al.* 2014).

In this paper, we review the current knowledge of PepMV evolutionary dynamics, genetic determinants of disease symptoms, detection methods and control strategies.

Evolutionary dynamics

PepMV is widely spread throughout the main tomato production areas around the world and its population is very diverse. PepMV isolates can be classified into five strains: European (EU), Peruvian (LP), southern Peruvian (PES), American (US1) and Chilean 2 (CH2), based on nucleotide sequence similarity (Hanssen *et al.* 2009; Moreno-Pérez *et al.* 2014). Over the last few years, dramatic changes in the population structure of the virus have been observed. Prevailing strains have been rapidly replaced by others.

After the first identification of the virus in Peru (LP strain) (Jones *et al.* 1980) the virus was next detected in the Netherlands in 1999 (EU strain) (van der Vlugt *et al.* 2000). The subsequent epidemic expansion of the virus rapidly covered Europe and North America (French *et al.* 2001; Mumford and Metcalfe 2001; Aguilar *et al.* 2002; Cotillon *et al.* 2002; Maroon-Lango *et al.* 2005; Ling 2007; Hanssen and Thomma 2010; Gómez *et al.* 2012). Initially, the virus population in Europe was rather homogeneous and contained isolates belonging to the EU strain. The nucleotide sequence similarity between the EU and the original LP populations was 96%, suggesting their common origin (Pagán *et al.* 2006; Hanssen and Thomma 2010). Within a short period of time, in Europe the EU isolates were largely replaced by CH2 isolates, which now prevail in the European PepMV population (Hanssen *et al.* 2008; Gómez *et al.* 2009; Tiberini *et al.* 2011). Although the CH2 strain has become dominant in Europe, in some countries the EU strain persists in the population in mixed infections with other strains. Mixed infections have been reported in Spain, Belgium and the Netherlands (Hanssen *et al.* 2008; Gómez *et al.* 2009).

In America the first PepMV isolates were identified in 2005 in the United States and classified as US1 and US2 strains. The last one was shown to be a recombinant (Maroon-Lango *et al.* 2005; Hasiów-Jaroszewska *et al.* 2010b). In 2007 in Chile, new PepMV isolates were described and classified into CH1 and CH2 strains (Ling 2007). These isolates shared only 80% sequence similarity with those representing the EU and LP strains. Also in the US, the population has shifted over time, with the first detection of the US1 strain (Maroon-Lango *et al.* 2005), subsequent predominance of the EU strain and later on a shift towards the CH2 strain (Ling 2008; Ling *et al.* 2013). Another shift was observed in Mexico in 2012, which resulted in a change in dominance from the CH2 strain to the US1 strain (Ling *et al.* 2013). The fifth strain, designated as PES, has recently been identified on wild tomatoes, *Solanum chilense* (Dunal) Reiche, *S. peruvianum* Zahlbr., *S. pimpinellifolium* L., and *S. pinnatifidum* Elliott in Peru in 2014 (Moreno-Pérez *et al.* 2014).

In Poland, in 2002 and 2007 isolates belonging to the EU strain were occasionally observed (Pospieszny *et al.* 2003, 2008; Pospieszny and Borodynko 2006). From 2005 until 2016 at the Department of Virology and Bacteriology (Institute of Plant Protection – National Research Institute), a collection of Polish PepMV isolates primarily belonging to the CH2 strain was obtained, revealing that currently there is a clear dominance of the CH2 strain in the Polish PepMV population. In contrast to the genetic homogeneity of the PepMV population, significant differences in host range and symptomatology were observed between these isolates. Based on the symptoms on the green parts of the plants, three main Polish pathotypes were distinguished: mild/wild-type, necrotic and yellowing (Pospieszny *et al.* 2008; Hasiów-Jaroszewska *et al.* 2009, 2013) (Fig. 1A, B, C). Most of the Polish PepMV isolates display only mild to moderate deformation and bubbling of the leaves in bio-assays, and are thus categorized as 'mild/wild-type'. However it should be noted that the name 'mild' is based on bio-assays and thus on leaf symptoms. They do not take into consideration the typical PepMV induced fruit marbling and discoloration which cannot be assessed in bio-assays. Most of the isolates described as 'mild/wildtype' based on leaf symptoms in this review do actually cause marbling and discoloration on tomato fruit and can thus cause significant losses to the Polish tomato growers. Next to these 'mild/wildtype' isolates, the first necrotic and yellowing isolates were found in 2007 and 2013, respectively. Yellowing and necrotic pathotypes induce severe symptoms on both the green plant parts and the tomato fruit. In contrast to other isolates, yellowing isolates caused interveinal leaf yellowing in seventeen tested varieties of tomato plants (data not shown). In addition, two potato varieties (Lord and Elanda) also displayed similar interveinal leaf yellowing symptoms upon inoculation with these isolates under greenhouse conditions (data not shown). It has been shown in earlier research that potato is not a main host of PepMV. However, viral disease symptoms, such as local necrotic lesions, mild systemic mosaic or mild systemic leaf chlorosis, were observed in certain susceptible varieties of potatoes after artificial inoculation with specific PepMV isolates (Blystad *et al.* 2015).

The occurrence of disease symptoms is associated with changes at the cellular level. Electron microscopy studies revealed characteristic changes in different compartments of various organelles (Jones *et al.* 1980; Alfaro Fernandez *et al.* 2010; Mathoiudakis *et al.* 2012; Minicka *et al.* 2015a). It has been shown that Polish necrotic isolates cause strong changes in the structure and function of individual organelles (i.e. mitochondria, chloroplasts, nuclei) seven days after inoculation. After inoculation with a necrotic isolate, severe damage occurs in the protoplasts, often resulting in shrinking and a separation from the cell wall, leading to plant cell death after 3–4 weeks (Minicka *et al.* 2015a). Such dramatic changes are not typi-

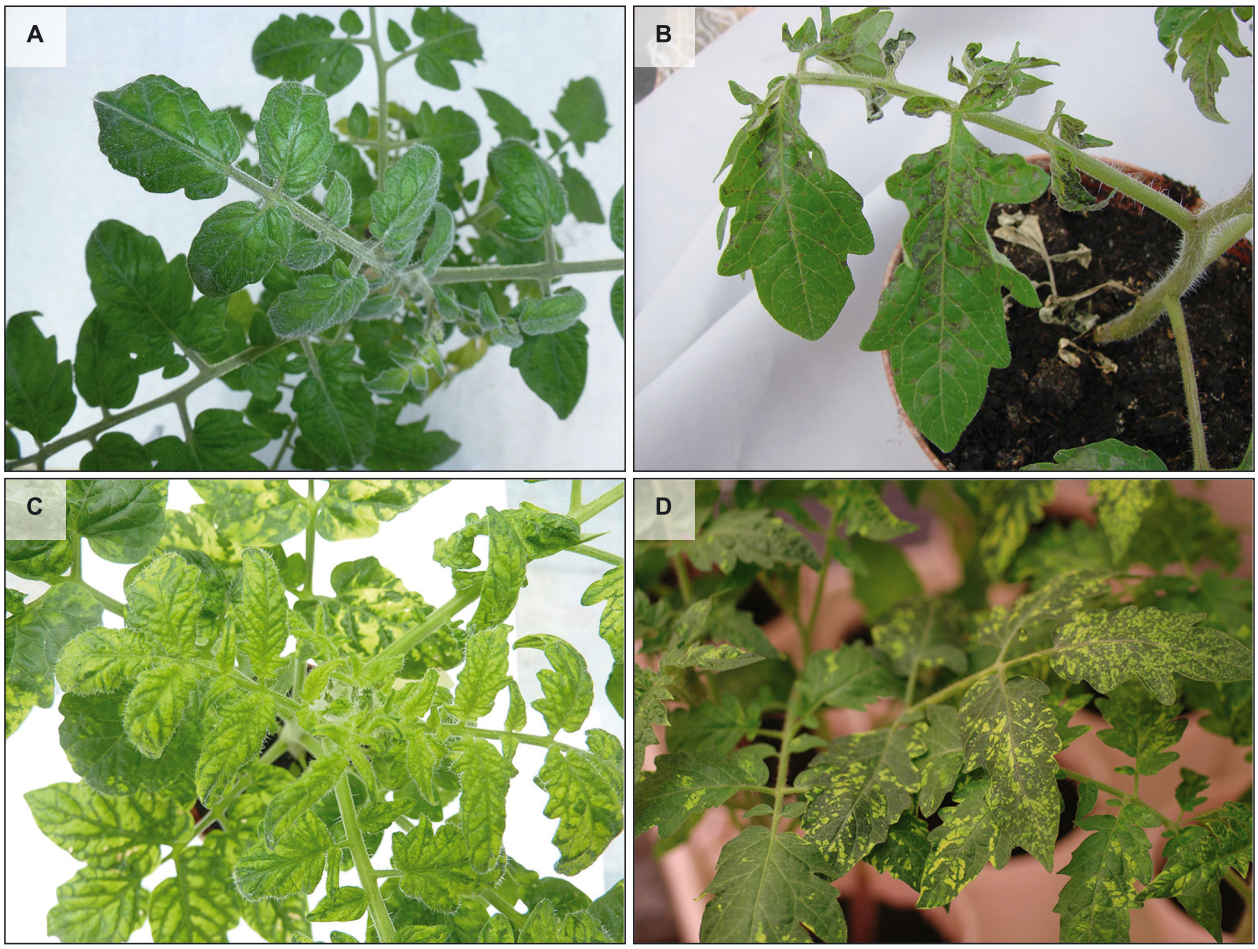

Fig. 1. Symptoms on tomato plants caused by different PepMV isolates: A – mild/wild-type (PepMV-P22); B – necrotic (PepMV-P19); C – interveinal yellowing (PepMV-P5-IY); D – graffiti (French isolate ST 11/019A)

cal for other PepMV variants. The mild pathotype does not cause changes in the structure of organelles, whereas the yellowing pathotype causes changes in the structure of the chloroplast and the nucleus 14 days after inoculation (Minicka *et al.* 2015a). The presence of PepMV infection was shown to be associated with increased activity of the rough endoplasmic reticulum resulting in extensive vesiculation. Formation of vesicular and membranous structures is associated with the secretion of proteins towards the cell wall and can be related with some defense processes (Minicka *et al.* 2015a).

Genetic determinants of disease symptoms

In recent years, detailed phylogenetic studies of the PepMV population have been conducted (Hasiów-Jaroszewska *et al.* 2010a; Gómez *et al.* 2012; Ling *et al.* 2013; Moreno-Pérez *et al.* 2014). It has been shown that nucleotide sequence similarity within strains is very high (about 95–99%), thereby forming homogeneous groups. Although different isolates belonging to a particular strain are genetically very similar, they cause very diverse disease symptoms on tomato. As described above, based on the symptoms on green plant parts in bio-assays three main pathotypes (mild/wild-type, yellowing and necrotic) have been observed in the virus population in Poland. Detailed phylogenetic analysis has shown that these different pathotypes can share 99% nucleotide sequence identity (Pospieszny *et al.* 2011). Single nucleotide substitutions in the genome were shown to be responsible for the development of deviant (necrotic and yellowing) disease symptoms (Hasiów-Jaroszewska *et al.* 2011, 2013). Necrotic symptoms are related to a single mutation in the gene encoding the TGB3, whereas three separate mutations in the gene encoding the CP were identified as individual determinants of similar interveinal leaf yellowing symptoms (Hasiów-Jaroszewska *et al.* 2008, 2013; Ortega-Parra *et al.* 2016a).

Single nucleotide substitution from $A_{199}AA$ to $G_{199}AA$ in position 199 nt (67 aa) in the gene encoding the TGB3, which resulted in a change of amino acid composition from K (lysine in wild-type isolates) to E (glutamic acid), is involved in the development of necrosis (Hasiów-Jaroszewska *et al.* 2011). This mutation was shown to affect virus accumulation, increasing it up to ten-fold (Hasiów-Jaroszewska *et al.* 2013; Sempere *et al.* 2016). The presence of this mutation appeared to be necessary but not enough to induce necrotic symptoms. Necrotic symptoms are only expressed when this mutation co-occurs with over expression of the RdRp-POL domain (Sempere *et al.* 2016). The presence and intensity of the symptoms also depend on the climate conditions and the tomato cultivar (Hasiów-Jaroszewska and Komorowska 2013; Sempere *et al.* 2016). The strongest necrotic symptoms occur at moderate temperatures of around 22–23°C, leading sometimes to plant death, whereas at higher temperatures (above 25°C) the

symptoms cannot even occur (Hasiów-Jaroszewska and Komorowska 2013; Sempere *et al.* 2016).

Other determinants of disease symptoms are located in the gene encoding the CP. The occurrence of interveinal leaf yellowing symptoms is correlated with three independent single nucleotide substitutions in positions 463 nt (155 aa), 497 nt (166 aa) and 706 nt (236 aa), respectively (Hasiów-Jaroszewska *et al.* 2013; Ortega-Parra *et al.* 2016a). The first mutation changes the codon from $G_{463}AA$ to $A_{463}AA$, resulting in an amino acid substitution from E (glutamic acid in wild-type isolates) to K (lysine). The second one converts the codon from $GA_{497}U$ to $GG_{497}U$, affecting amino acid substitution from D (aspartic acid in wild-type isolates) to G (glycine). The third mutation changes the codon from $G_{706}AA$ to $C_{706}AA$ resulting in amino acid substitution from E (glutamic acid in wild-type isolates) to Q (glutamine). Surprisingly, it has been shown that these yellowing symptoms are not affected by temperature conditions (Hasiów-Jaroszewska *et al.* 2013).

Besides interveinal yellowing symptoms, severe mosaic patterns consisting of irregular, bright yellow areas on tomato leaves (referred to as 'graffiti' plants) have also been reported (Fig. 1D). After several weeks, affected plants seemed to have recovered and no yellowing or severe mosaic symptoms were visible anymore in the upper parts of the plants. Sequencing of CP clones from plants or plant parts with the severe mosaic symptoms resulted in a mixture of wild-type (mild isolate) and mutated sequences (yellowing isolate), while sequencing of CP clones from the green heads of recovered plants resulted in only wild-type sequences. Severe mosaic symptoms ('graffiti') could be reproduced by inoculating an artificial 1 : 1 mixture of RNA transcripts from the wild-type and the mutated infectious clones. The gradual recovery of the plants, which coincided with the disappearance of the yellowing mutations, suggests that selection pressure acts to the advantage of the wild-type virus. Experiments with the wild-type and mutated infectious clones have shown that back-mutation towards the wild-type sequence, rather than a difference in accumulation speed or efficiency, causes the disappearance of the yellowing symptoms.

Finally, it has been shown that some of these genetic determinants of PepMV disease symptoms are not only specific to the CH2 strain (Hasiów-Jaroszewska and Borodynko 2012).

Multifaceted capsid protein

Recently, many studies have been devoted to the nature, structure and function of the CP of the plant viruses. In plant RNA viruses, the CP is important at several stages of the viral infection, starting from the early stage of disassembly or uncoating to the encapsidation of the new viral nucleic acids. The presence of the CP is also necessary in translation, replication, suppression of RNA silencing, cell-to-cell movement and symptom development (Callaway *et al.* 2001; Verchot-Lubicz 2005; Bol 2008).

As described above, alterations in the gene encoding the CP of PepMV cause the development of interveinal leaf yellowing symptoms (Hasiów-Jaroszewska *et al.* 2013). This region of the genome interacts with the tomato heat shock protein cognate 70 (Hsc70) (Mathioudakis *et al.* 2012). Hcs70 is a conserved molecular polypeptide chaperone involved in different processes such as protein refolding, transport to and across membranes, complex assembly and receptor signaling (Bukau and Horwich 1998; Sung *et al.* 2001; Mayer and Bukau 2005; Wang *et al.* 2009). Hcs70 was also reported to affect virus replication, accumulation, movement, and protein folding (Whitham *et al.* 2003; Aparicio *et al.* 2005; Chen *et al.* 2008; Nagy *et al.* 2011). The PepMV CP-Hsc70 complex was observed in the phloem cells of infected tomato plants (Mathioudakis *et al.* 2012). This suggests that a specific interaction between the Hsc70 chaperone and the viral CP occurs during viral infection and replication (Mathioudakis *et al.* 2012). This might also be linked with long distance transport of the virus.

Recently, it has been found that the 3'-terminal region of the genome of PepMV functions as a virulence factor (Duff-Farrier *et al.* 2015). Chimeric infectious clones of EU (mild) and CH2 (aggressive) isolates were constructed and used to infect *S. lycopersicum*, *N. benthamiana* and *D. stramonium* plants. Increased symptom severity was exclusively observed in *N. benthamiana* plants inoculated with such chimeric clones in comparison to those infected with the EU wild-type. This study showed that a pathogenicity determinant is located between amino acids 11 and 26 of N-terminal region of the CP gene and is host specific. Further studies are required to investigate which host factors are involved in interaction with the N-terminal CP region during the infection. A similar role of the N-terminal region of the CP in the induction of symptoms has also been observed in other plant viruses, such as the *Potexvirus Bamboo mosaic virus* (BaMV) (Lan *et al.* 2010). It has been found that the N-terminal 35 amino acids of the CP containing a glycine rich motif (GRM) are determinants of the symptoms of BaMV.

Based on the multifunctional character of the CP and its essential role in many processes, localization experiments of CP proteins of different isolates at different stages of infection have been performed (Minicka *et al.* 2015a). The labeling of the CP proteins was strong, especially in plant conductive elements, such as xylem vessels, immature and mature sieve elements, phloem companion cells and mesophyll cells, confirming fast movement of the virus. Plant viruses use different mechanisms for cell-to-cell and long-distance (phloem) movement. Some RNA plant viruses use only one mechanism, whereas others use both mechanisms on infected plants (Leisner *et al.* 1992; Carrington *et al.* 1996; Roberts *et al.* 1997; Medina *et al.* 2006; Wan *et al.* 2015). The localization experiments confirmed that PepMV uses both mechanisms for movement within the tomato plants (Minicka *et al.* 2015a).

PepMV evolution

Knowledge about the rate and spectrum of spontaneous mutations is essential for the analysis of evolutionary dynamics of viruses. It has been shown that PepMV, which creates quasispecies populations, displays high mutation rate during replication (Hasiów-Jaroszewska

et al. 2010a). Viral quasispecies are collections of closely related viral genomes (also known as mutant clouds or mutant swarms), which arise in infected organisms during replication process (Andino and Domingo 2015). These closely related variants are subject to continuous processes of competition and selection (Domingo *et al.* 2012). The mean PepMV molecular evolution rate has been estimated at 5.570 × 10−3 substitutions/site/year (Gómez *et al.* 2012). High mutation rate results in a higher potential for horizontal transmission in host populations and therefore increases the effective virus population size (Duffy *et al.* 2008). Recently, studies on PepMV evolution have been performed under experimental conditions (Minicka *et al.* 2015b). To study PepMV genetic evolution, a Polish mild isolate (PepMV-P22) was serially passaged (20 times) in five lineages of different hosts (three varieties of tomato and *D. inoxia*). At the end of the experiments the TGB3 and CP genes of all evolutionary lineages were amplified and sequenced for sequence comparison. Results of this study show that PepMV tends to incorporate mutations that may strongly affect the symptomatology. During long-term passaging in different hosts, a high number of viral variants have been created. A positive correlation between nucleotide diversity and severity of symptoms was observed, with more diverse populations being more aggressive than populations with lower polymorphism (Minicka *et al.* 2015b). In an earlier study focusing only on the TGB1 gene, similar results were found, with virus aggressiveness being positively correlated with quasispecies complexity (Hasiów-Jaroszewska *et al.* 2010a).

In the work of Minicka *et al.* (2015b), significant differences in viral evolution rates were observed between the different hosts, especially between *D. inoxia* and tomato. At the end of the experiment, the virus lineages evolved in *D. inoxia* were genetically less diverse than the lineages in tomato. After 18 passages, necrotic symptoms were observed in two independent evolutionary lines of the tomato cultivar Beta Lux. Sequencing of the TGB3 region of PepMV isolated from these plants revealed the presence of A_{199}AA-G_{199}AA mutation typical for necrotic variants. The emergence of this mutation under experimental conditions may indicate either its persistence in the virus population or adaptive evolution. It has been shown that depending on the host, mutations in the virus genome may occur with higher or lower frequency, resulting in more or less variability (Rodríguez-Cerezo and García-Arenal 1989; Pita *et al.* 2007; Wallis *et al.* 2007; Vozárová *et al.* 2013).

The distribution of mutational fitness effect (DMFE) of nonsynonymous mutations on a mild isolate of PepMV from the Chilean 2 genotype (PepMV-P22) was also explored (data not shown). It has been found that among introduced mutations 80% were neutral or beneficial, indicating a high degree of robustness against point mutations of PepMV-P22 genome. It suggests the high evolutionary potential of PepMV, which makes it a very dangerous pathogen.

Detection methods

The rapid appearance of new, sometimes more virulent viral variants, such as the Polish necrotic variant – PepMV-P19, can be problematic for commercial tomato production. The efficient mechanical transmission of the virus through contaminated tools, hands, clothes and by direct plant-to-plant contact (Jones *et al.* 1980; Wright and Mumford 1999; Spence *et al.* 2006) makes it difficult or even impossible to control the spread of such new variants, especially since they might already have been spread before the symptoms appear. In an attempt to better manage this disease in commercial tomato production, many diagnostic methods have been developed in recent years. Because of the very diverse phenotypes (from asymptomatic to severe necrosis), diagnosis based on symptoms is not reliable. For routine detection of the virus on infected plant material, standard enzyme-linked immunosorbent assay (ELISA) procedures and qPCR assays can be used (Ling *et al.* 2007; Hasiów *et al.* 2008; Gutiérrez-Aguirre *et al.* 2009; Hanssen *et al.* 2010a). In order to identify pathotypes, which often share a 99% sequence similarity, specific primers for reverse transcription-polymerase chain reaction (RT-PCR) or RT qPCR can be used (Pagán *et al.* 2006; Ling *et al.* 2007). A method based on real-time PCR with melting curve analysis (high resolution melting – HRM) was developed to differentiate between necrotic and wild-type isolates based on a single mutation in the TGB3 gene (Hasiów-Jaroszewska and Komorowska 2013).

Classical molecular methods can be used only under laboratory conditions with appropriate equipment. One of the techniques which can be performed under field or greenhouse conditions is the reverse transcription loop-mediated isothermal amplification (RT-LAMP). LAMP is a molecular diagnostic method that allows a precise detection of the virus in infected material. The amplification reaction is run at a constant temperature. By adding a fluorescent reagent post reaction, the extra step of visualization by gel electrophoresis is not necessary. Moreover, this method is characterized by a high level of sensitivity and specificity. Many plant viruses can already be identified using this approach (Peng *et al.* 2012; Zong *et al.* 2014). Universal PepMV and strain specific RT-LAMP assays have been developed and successfully applied for PepMV detection by Hasiów-Jaroszewska and Borodynko (2013) and Ling *et al.* (2013), respectively.

Control strategies

Designing new strategies to protect plants against viruses is essential for crop production. Due to severe outbreaks of PepMV in greenhouse tomato production in many European countries in recent years, the first plant protection product against PepMV damage was developed in Belgium. The method is based on the principle of cross-protection, where a mild isolate is used as a protective agent against more aggressive strains. The principle of cross-protection has been optimized and applied for many economically important pathogens, such as *Tomato mosaic virus* (ToMV) (Rast 1972; Oshima *et al.* 1975; Fletcher 1978), *Papaya ringspot virus* (PRSV) (Wang *et al.* 1987), and *Cassava mosaic virus* (Owor *et al.* 2004).

The potential of different mild isolates of PepMV to protect plants against more aggressive isolates using cross-protection was examined (Hanssen *et al.* 2010b).

Three mild isolates belonging to different strains were tested in a plastic tunnel trial. Challenge inoculation was done with an aggressive isolate belonging to the predominant CH2 strain. The experiments showed that only a specifically selected, mild CH2 isolate provided efficient protection (Hanssen *et al.* 2010b). In the other cases, the symptoms were enhanced as a result of the co-infection with two strains (mixed infection). Further research and optimization has resulted in a vaccination strategy using this well-defined, characterized and stable CH2 mild isolate. The vaccine was named PMV-01 and has been registered in the EU as a plant protection product under EU regulation 1107/2009. Since a couple of years, this strategy has been successfully applied to control the damage of PepMV in greenhouse tomato crops in different countries (Ortega-Parra *et al.* 2016b).

Similar cross-protection experiments have been performed with Polish isolates from the CH2 strain (Hasiów-Jaroszewska *et al.* 2014). Necrotic and yellowing isolates, PepMV-P19 and PepMV-P5, respectively, were used as challenge isolates, whereas the mild isolate PepMV-P22 was used as a protective isolate. In the plants challenged with the yellowing isolate, yellowing symptoms were not observed on infected plants, even 28 days post inoculation (dpi). In the plants challenged with PepMV-P19, necrotic changes on leaf blades appeared after 14 days. Therefore, it was concluded that this Polish mild isolate cannot be used for protection (Hasiów-Jaroszewska *et al.* 2014). The fact that the cross-protection was overcome by this necrotic variant PepMV-P19 is probably associated with a faster rate of replication and greater accumulation of PepMV-P19 than the mild isolate PepMV-P22.

In summary, PepMV is a very important pathogen in greenhouse tomato production, which has rapidly spread over recent years despite certain protective regulations taken at the EU level. Moreover, the initial structure of the virus population has been completely changed and more virulent variants have appeared under natural conditions. Characterization of the genetic variation of viral populations provides relevant information on the processes involved in virus evolution and epidemiology and it is crucial for designing reliable diagnostic tools and developing efficient and durable disease control strategies. Due to the fast rate of replication and easy transmission of the virus, new outbreaks can be expected in the future. In this context, it seems necessary to continue expanding our knowledge of PepMV.

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