Genetic stability study of different isolates of *Pepino mosaic virus* (including the mild isolate from the vaccine PMV®-01) through several passages in tomato and further cloning and sequencing of particular genome regions

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INTRODUCTION

Pepino mosaic virus (PepMV) is included in the European Plant Protection Organization list as one of the most important tomato pathogens worldwide. PepMV belongs to the *Potexvirus* genus in the *Alphaflexivirdae* family. The genome consists of one single strand positive-sense RNA approximately 6.4 kb long, flanked by two short untranslated regions (UTRs) with a cap at the 5' and a poly (A) tail at the 3' end of the genomic RNA. The genome contains five open reading frames (ORFs): ORF1 encodes a putative viral polymerase (RdRp) of 164kDa, containing a methyltransferase, a nucleoside triphosphate (NTP)-binding and polymerase

motifs. ORFs 2-4 encode the movement proteins (TGBp 1-3) of 26, 16 and 9 kDa, respectively, while ORF5 encodes the 25 kDa coat protein (CP) (Fig. 1) (Maroon-Lango et al., 2005; Ling, 2007; Hasiów et al., 2008).

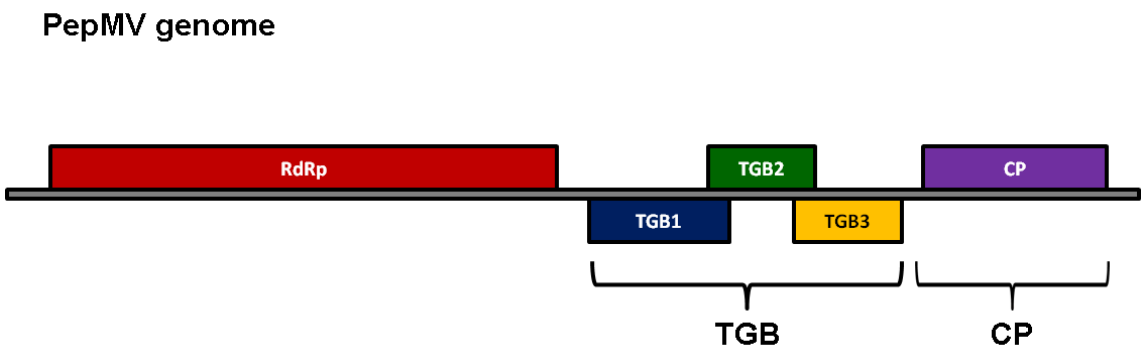

Figure 1. The schematic overview of the PepMV genome organization, displaying the encoded gene products. The regions that were sequenced and analyzed in this study are marked (TGB and CP).

Based on the phylogenetic analysis four different genotypes have been described so far, i.e. European (EU), Peruvian (LP), American (US1) and Chilean 2 (CH2) (Hanssen et al., 2009). The shift in the prevalent genotype population of PepMV from the genotype EU to CH2 occurred in Europe and North America and currently the CH2 genotype is the most widespread (Ling, 2013). Isolates belonging to the CH2 genotype share a very high nucleotide sequence similarity ranging from 98 to 100%. It has also been shown that PepMV exists as a quasispecies cloud and that single point mutations affect virus virulence (Hasiów-Jaroszewska et al., 2011; Hasiów-Jaroszewska et al., 2010). It has been recently observed that the rate of PepMV molecular evolution is on average 5.570×10^{-3} substitutions/site/year (Gómez et al., 2012). This value is higher than the rates reported recently for other plant RNA viruses, which may suggest that PepMV evolves faster than other plant viruses.

It has been shown that single nucleotide substitutions play a role in the development of symptoms on tomato plants. The K67E substitution in the TGB3 caused the development of necrotic symptoms on tomato plants (Hasiów-Jaroszewska et al., 2009, Hasiów-Jaroszewska et al., 2011). It has also been shown that two separate mutations (E155K and D166G) in the

coat protein result in the development of yellowing symptoms on tomato plants (Hasiów-Jaroszewska et al., 2013).

Severe yield and quality losses clearly show the need to find new methods to protect plants against this virus (Soler-Aleixandre et al., 2005). As no resistant tomato cultivars exist and PepMV is the most prevalent virus in tomato, cross-protection may offer an alternative strategy to reduce economic losses. Cross-protection is a phenomenon, in which infection of a plant with a mild virus isolate protects the plant from disease caused by a subsequent infection with related, more severe isolates (Gal-On et al., 2006). A good cross-protection isolate should be mild, genetically (genotype) and biologically (phenotype) stable and accumulate to sufficient levels in the plant tissue.

The goal of this research was to determine the genetic stability of the mild PepMV isolate 1906 from the vaccine PMV®-01 in comparison with other, randomly selected, more aggressive isolates.

MATERIALS AND METHODS

10.1.c Wob juncto 63.2.a Vo 1107/2009 juncto 39.2.a Vo 178/2002

[redacted]

10.1.c Wob juncto 63.2.a Vo 1107/2009 juncto 39.2.a Vo 178/2002

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10.1.c Wob juncto 63.2.a Vo 1107/2009 juncto 39.2.a Vo 178/2002

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RESULTS AND DISCUSSION

Variability within the genotype

The genetic diversity within each PepMV isolate [redacted], was expressed by the mean Hamming distance. The mean Hamming distances were calculated both for the nucleotide and amino acid sequences of the TGB1, TGB2, TGB3 and CP genes (Table 1 and 2). The TGB region consists of three overlapping genes: TGB1 (704 nt), TGB2 (369 nt), and TGB3 (246 nt). The CP gene is 711 nt long. All mutations observed in the PepMV populations were substitutions; no insertions and deletions were detected.

Table 1. Hamming distances calculated based on nucleotide sequences

Isolate	Hamming distance			
	TGB1	TGB2	TGB3	CP
1906	[redacted]	[redacted]	[redacted]	[redacted]
13-029A	1.58	1.42	0.49	2.38
B-361	1.82	0.91	0.33	2.57
PVU	2.72	0.83	0.25	1.5
CH2-agr	1.84	1.05	0.5	1.48

Table 2. Hamming distances calculated based on amino acid sequences

Isolate	Hamming distance			
	TGB1	TGB2	TGB3	CP
1906	[redacted]	[redacted]	[redacted]	[redacted]
13-029A	0.83	0.83	0.25	1.07
B-361	1.08	0.74	0.25	1.08
PVU	1.23	0.75	0.17	0.5
CH2-agr	0.82	0.80	0.33	0.99

The analysis of the TGB1 sequences shows that more aggressive isolates create a higher number of variants in this gene and were characterized by higher Hamming distances (1.58-2.72) in comparison to the asymptomatic isolate 1906 from PMV®-01 [redacted] These results are in line with a former study we performed (Hasiów-Jaroszewska et al., 2010), where we showed that isolates which induced more severe symptoms displayed a higher variability of the TGB1 gene, whereas the mild ones were usually characterized by a lower quasispecies complexity for this gene. This difference in variability of TGB1 sequences between

aggressive and mild viral isolates might by connected with the role of this gene product in systemic movement and in RNA silencing. It has been shown that the Potexvirus TGB1 protein is an RNA silencing inhibitor (Senshu et al., 2009).

The Hamming distance calculated for TGB2 was similar for all tested isolates (0.83-1.05) with the exception of 13-029A which showed slightly higher value 1.42. Hamming distance calculated based on amino acid sequences ranged from 0.74-0.88 for all the isolates tested in this experiment.

The Hamming distance obtained for TGB3 ranged from 0.25 to 0.57. In the isolate 1906 a higher Hamming distance [redacted] was obtained. However, this was a consequence of the presence of different point mutations in position [redacted]. However, these point mutations were silent and the Hamming distance calculated based on amino acid sequences was equal to zero.

The highest level of diversity in genetic stability was noticed for the CP gene where Hamming distance for nt sequences ranged from 1.48 to 2.57. Here the asymptomatic isolate 1906 had an intermediate variability: two isolates (CH2-PVU and CH2-ag) had a lower variability and two isolates had a higher variability.

Variability within the phenotype

For each isolate, the symptoms were observer after each passage. After eight passages, we did not observe any significant changes in the symptoms induced by the different isolates. Isolate B-361 induced severe malformation and mosaics on the tomato leaves during the whole experiment. The isolates CH2-PVU, CH2-agr and 13-029A caused severe malformation of leaves and a growth reduction of the plants, also until the last passage. However, the plants infected with isolate 1906 from PMV-01 remained completely asymptomatic until the last passage.

Conclusions:

[redacted] performed in tomato plants, cultivar Tricia, did not affect the symptoms induced by isolate 1906 from PMV-01. All plants infected with isolate

1906 from PMV-01 remained asymptomatic until the last passage. The mean Hamming distance calculated based on nucleotide and amino acid sequences differed depending on the region of the genome. More aggressive isolates had a higher genetic variability in the TGB1 gene, the mild isolate 1906 thus being the most stable isolate for this gene. The analysis of the CP sequences revealed a slightly different situation for the CP gene, in which the asymptomatic isolate 1906 had an intermediate variability: two isolates had a lower variability and two isolates had a higher variability.

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Approbation of the report

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Signature of the study director

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