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CHARACTERIZATION OF TWO DISTINCT *PEPINO MOSAIC VIRUS* ISOLATES FROM TOMATO IN LITHUANIA

Marija Žižytė*, Donatas Šneideris, Irena Zitikaitė, Laima Urbanavičienė, Juozas Staniulis

Nature Research Centre, Institute of Botany, Žaliųjų Ežerų Str. 49, LT-08406 Vilnius, Lithuania
* Corresponding author. E-mail: marija.zizyte@botanika.lt

Abstract

Žižytė M., Šneideris D., Zitikaitė I., Urbanavičienė L., Staniulis J., 2013: Characterization of two distinct *Pepino mosaic virus* isolates from tomato in Lithuania [Kiauliauogės mozaikos viruso (*Pepino mosaic virus*) dviejų skirtingų genotipų aptiktų pomidoruose Lietuvoje charakteristika]. – Bot. Lith., 19(1): 00–00.

Two isolates of *Pepino mosaic virus* (PepMV) from tomato plants grown in different commercial greenhouses in Lithuania were characterized by coat protein (CP) gene sequence analysis. Comparison with other PepMV isolates from the GenBank database showed that both Lithuanian PepMV isolates share 78.3% nucleotide identity and belong to two distinct EU and CH2 genotypes of PepMV. This is the first report on characterization of two PepMV genotypes detected in Lithuania.

Keywords: *Pepino mosaic virus*, potexvirus, genotypes, phylogenetic analysis.

INTRODUCTION

Pepino mosaic virus (PepMV, genus *Potexvirus*, family Alphaflexiviridae) was originally described on pepino (*Solanum muricatum*) in Peru in 1974 (Jones et al., 1980). In Europe, PepMV was first detected in 1999, in tomato crops (*Solanum lycopersicum*) in the Netherlands (Vlugt van der et al., 2000) and the UK (Mumford & Metcalfe, 2001). Since then the virus has become a major disease of tomato crops, causing significant yield losses worldwide (van der Vlugt et al., 2000; Mumford & Metcalfe, 2001; Cotillon et al., 2002; Maroon-Lango et al., 2005; Pagan et al., 2006; Hasiow et al., 2008; Hanssen et al., 2008). PepMV is highly contagious and can rapidly spread by mechanical means to young tomato seedlings. Although the efficiency of seed transmission is low, the highly infectious nature of PepMV implies a substantial risk associated with tomato seeds harvested from an infected crop (Córdoba-Sellés et al., 2007; Hanssen et al., 2010; Hanssen & Thomma, 2010). Therefore, seed might play a role in long distance spread of the virus. The virus causes a wide range of symptoms including fruit marbling and flaming, nettle heads, leaf mosaics, dwarfing, leaf distortions and yellow leaf spots. PepMV infection can significantly reduce the fruit quality (Roggero et al., 2001; Spence et al., 2006).

PepMV particles are filamentous and its genome consists of a positive, single-stranded RNA of about 6.5 kb. The polyadenylated RNA possesses a cap structure at the 5' end and contains five open reading frames (ORFs): ORF1 encodes for replication-associated proteins; ORFs 2–4 encode the triple gene block (TGB) proteins, which are essential for virus movement; and ORF5 encodes the coat protein (CP) (Mumford & Metcalfe, 2001; Aguilar et al., 2002; Cotillon et al., 2002; Maroon-Lango et al., 2005; Pagan et al., 2006; Ling, 2007; Hasiow et al., 2008). The virus has a high level of genetic diversity. Originally all PepMV isolates identified in Europe shared a high nucleotide sequence homology and were classified as European tomato genotype (EU). Lopez et al. (2005) have isolated PepMV from *Lycopersicon*

peruvianum and now this isolate is considered as the Peruvian genotype (LP) of PepMV. Since 2005, three divergent genotypes have been identified in tomato (Maroon-Lango et al., 2005; Ling, 2007). To date, four genotypes of PepMV have been identified: European (EU), Peruvian (LP), North American (US1) and Chilean (CH2) (Hanssen & Thomma, 2010; Papayiannis et al., 2012; Gómez et al., 2012). EU and CH2 genotypes are most common in Europe and it seems that CH2 genotype is now predominant and has largely replaced EU genotype in commercial tomato production (Hanssen et al., 2008; Pospieszny et al., 2008; Hanssen et al., 2010).

PepMV isolates have been found in tomato fruits obtained from supermarkets and imported to Lithuania from Spain, the Netherlands and Poland. In 2010 and 2011, we also investigated impact of PepMV on local tomato production in Lithuania. Over these two years, one hundred thirty one symptomatic samples of tomato leaves and fruit were collected and analysed. Two isolates of the virus (PepMV-NV and PepMV-KK) were found in two different commercial greenhouses (Staniulis et al., 2012). This paper presents nucleotide sequence analysis of full length coat protein (CP) gene of these two Lithuanian PepMV isolates.

MATERIALS AND METHODS

Virus sources, serological identification and mechanical inoculation. Virus sources were detected on tomato plants obtained from local tomato production in Lithuania. All samples were tested using double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) with specific antibodies (DSMZ GmbH, Germany) as described by Clark & Adams (1977). Tests were considered to be positive if the UV absorbance of investigated sample was equal or greater than three times the absorbance of negative control (healthy plant).

DAS-ELISA test revealed that two tomato samples from different locations were infected with PepMV. Isolate PepMV-NV was obtained from tomato plant exhibiting mild yellow leaf spotting, isolate PepMV-KK was obtained from tomato fruits with marbling symptoms. Our previous host range studies showed *Nicotiana benthamiana* to be good propagation plant for both PepMV isolates (Staniulis et al., 2012). Mechanical inoculation of indicator plants (*N. benthamiana* or tomato plants) was performed using crude sap extracted from infected plant samples. Leaves of infected plants were ground in sodium phosphate buffer (pH 7.0–7.2) and rubbed on experimental plant leaves dusted with carborundum for virus propagation and multiplication. Inoculated plants were maintained in the greenhouse (conditions: 18–30ºC, under a long day). PepMV infectivity was confirmed by the development of typical disease symptoms on *N. benthamiana* and tomato plants. DAS-ELISA confirmed the presence of the virus.

Reverse transcription-polymerase chain reaction (RT-PCR) and sequence analysis. Total RNA extraction was performed with TRIzol Reagent (Invitrogen, USA) according to the protocol as described by Chomczynski & Sacchi (1987).

Extracted RNA was used for virus detection and identification by RT-PCR. Extracts from healthy plants were used as negative control. Primers for PepMV detection were PepTGB-F: 5'- CACACCAGAAGTGCTTAAAGCA – 3' and PepUTR-R: 5' – CTCTGATTAAGTTTCGAGTG – 3' (Mumford & Metcalfe, 2001). Amplification was performed in TProfessional Thermocycler (Biometra, Germany) using double-step RT-PCR method. The first strand cDNA synthesis was carried out at 37ºC for 60 min and 70ºC for 10 min using PCR mix of 20 µM specific reverse primer, RT 5× buffer, 10 mM dNTP, Ribonuclease Inhibitor (40 $U\mu l^{-1}$) and RevertAid™ reverse transcriptase (200 $U\mu l^{-1}$) (Fermentas, Lithuania). DNA amplification reaction was performed in mixtures containing 2 µl of each primer (25 µM), 5 µl 10X Taq Buffer, 3 µl $MgCl_2$ (25 mM), 1 µl dNTP mix (10 mM), 0.25 µl Taq polymerase (5 $U\mu l^{-1}$) (Fermentas, Lithuania), 4 µl cDNA and DEPC H_2O up to a total volume of 50 µl. The following cycling scheme was used: 94ºC – 4 min, followed by 40 cycles of [94ºC – 1 min, 55ºC – 2 min, 72ºC – 2 min] and 72ºC – 10 min.

Amplified DNA fragments were analysed by electrophoresis in 1.5% agarose gel, stained with etidium bromide and visualized in BioDocAnalyze (Biometra, Germany) gel documentation system using GeneRuler™ 50 bp DNA Ladder marker (Fermentas, Lithuania).

Specific 845 bp length PCR products were purified

using a DNA extraction kit (Fermentas, Lithuania) according to the instruction of manufacturer before sequencing at Macrogen (Korea). Sequencing was carried out in two directions. Obtained nucleotide sequences were submitted to the GenBank database (accession numbers JQ979169 for PepMV-NV and JQ979170 for PepMV-KK). Nucleotide sequences were compared with sequences available in the GenBank using BLAST. Alignments were performed using DNASTAR7 and MEGA4.1 (by Kimura's two-parameter method) (Tamura et al., 2007) computer programmes. Phylogenetic analysis was carried out by neighbour-joining method with bootstrap values based on 1000 pseudoreplicates.

RESULTS AND DISCUSSION

The results of the study showed that Lithuanian PepMV isolates differ from each other. Phylogenetic analysis grouped Lithuanian isolates into two distinct EU and CH2 genotypes (Fig. 1 and Table 1). Both Lithuanian PepMV isolates shared 78.3% nucleotide sequence for the CP gene. Lithuanian PepMV-NV isolate (Accession no. JQ979169) belongs to the EU genotype and showed the highest (99.7%) nucleotide identity to the isolates from the Netherlands, the USA, France and Poland (Accession no. FJ940223, JQ314457, AJ438767 and EF408822, respectively). The identity of PepMV-NV isolate with other geno-

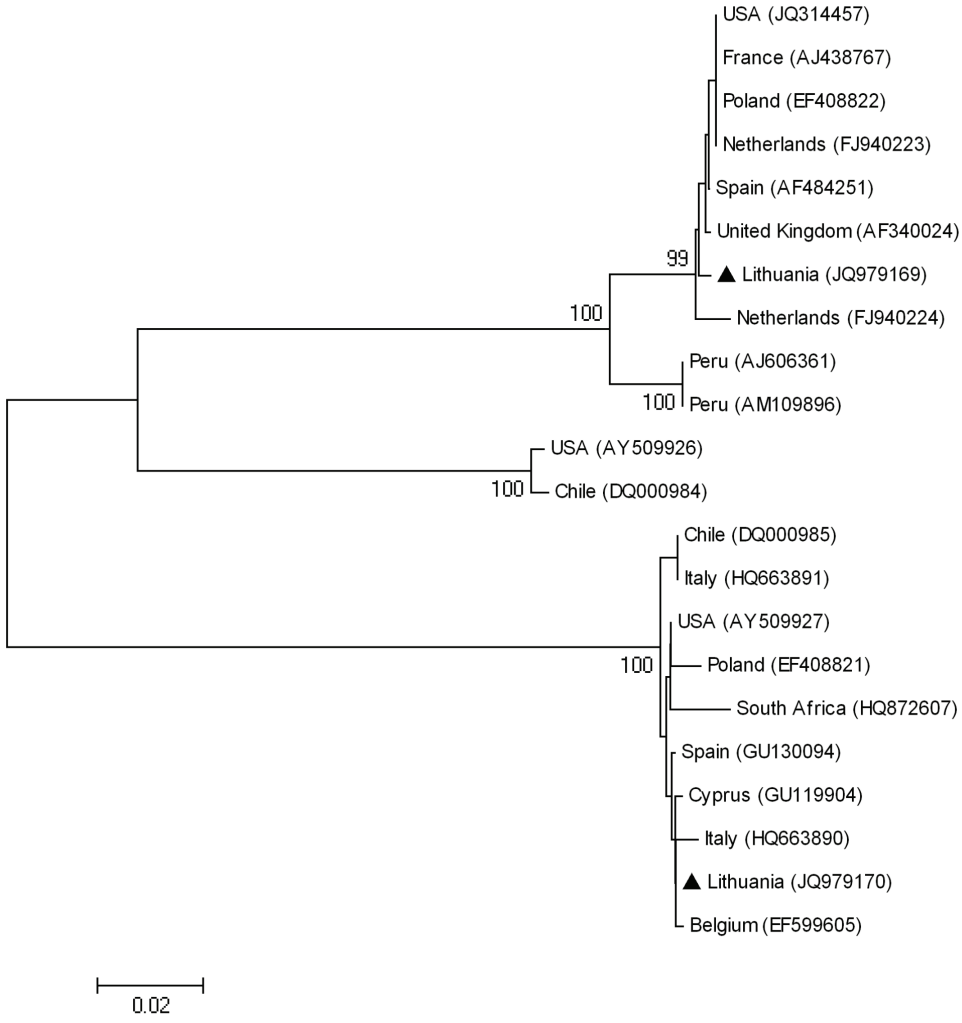

Fig. 1. Phylogenetic tree of *Pepino mosaic virus* based on coat protein full gene sequences, constructed with MEGA4.1 using neighbour-joining algorithm. Subjected to 1000 bootstrap replicates, numbers at branching points indicate bootstrap values (only values > 90% are shown). The horizontal branch lengths are proportional to the genetic distances. Lithuanian isolates are marked with ▲ symbol

Table 1. Genotypes of *Pepino mosaic virus* (PepMV) isolates and GenBank accession numbers of PepMV encoded coat protein gene

Country	Isolate name	Accession number	Genotype	Reference
Belgium	220606A1	EF599605	CH2	Hanssen et al., 2008
Chile	Ch1	DQ000984	US1	Ling, 2007
	Ch2	DQ000985	CH2	Ling, 2007
Cyprus	CY-PepMV-Odou	GU119904	CH2	Papayiannis et al., 2012
France	–	AJ438767	EU	Cotillon et al., 2002
Italy	SAR09	HQ663890	CH2	Tiberini et al., 2011
	SIC1-09	HQ663891	CH2	Tiberini et al., 2011
Lithuania	PepMV-NV	JQ979169	EU	This study
	PepMV-KK	JQ979170	CH2	This study
Netherlands	EU-tomato	FJ940223	EU	Vlugt van der et al., 2002
	DB1	FJ940224	EU	Vlugt van der et al., 2002
Peru	LP-2001	AJ606361	LP	Lopez et al., 2005
	SM.74	AM109896	LP	Pagan et al., 2006
Poland	PK	EF408821	CH2	Hasiow et al., 2008
	SW	EF408822	EU	Pospieszny et al., 2008
Spain	Sp-13	AF484251	EU	Aguilar et al., 2002
	Mu-4-08	GU130094	CH2	unpublished
South Africa	–	HQ872607	CH2	Carmichael et al., 2011
United Kingdom	–	AF340024	EU	Mumford & Metcalfe, 2001
USA	EU_CAHN8	JQ314457	EU	Li et al., 2012
	US1	AY509926	US1	Maroon-Lango et al., 2005
	US2	AY509927	CH2	Maroon-Lango et al., 2005

types was: 96.8% to the LP genotype (Accession no. AJ606361), 83.1% to the US1 genotype (Accession no. AY509926) and 78.5% to the CH2 genotype (Accession no. EF599605). Whereas Lithuanian PepMV-KK isolate (Accession no. JQ979170) was assigned to the CH2 genotype. PepMV-KK showed the highest nucleotide sequence similarity (99.9%) to isolates from Belgium (Accession no. EF599605) and Spain (Accession no. GU130094). CP gene translated amino acid sequence of PepMV-KK with the aforementioned isolates from Belgium and Spain was 100% identical. The identity of PepMV-KV isolate was 78.6% with the isolate of the LP genotype (accession no. AJ606361), 80.6% with the isolate of the US1 genotype (accession no. AY509926) and 78.2% with the isolate of the EU genotype (accession no. FJ940223).

In recent years increasing incidence of PepMV CH2 genotype has been observed worldwide. PepMV CH2 has spread and become predominant in Poland (Hasiów-Jaroszewska et al. 2010), Spain (Gómez et al., 2009; Gómez et al., 2012), Belgium (Hanssen et al., 2008) and North America (Ling et al., 2008). This could be related to a more rapid spread and accumulation of PepMV CH2 genotype than the EU genotype within a crop (Hanssen et al., 2008). More recently the CH2 genotype was detected in Greece (Efthimiou et al., 2011), Italy (Tiberini et al., 2011), South Africa (Carmichael et al., 2011) and Cyprus (Papayiannis et al., 2012). It seems that the incidence of PepMV, especially the CH2 genotype, is increasing worldwide and commercial tomato growers might unwillingly provide possibilities for the virus to cross international boundaries. Virus can be acquired with imported tomato seedlings, seeds or fruit and then mechanically spread into the environment.

CONCLUSIONS

Lithuanian *Pepino mosaic virus* isolates differ from each other and belong to two distinct EU and CH2 genotypes of PepMV. Since no resistant varieties are available at present, further studies are necessary to determine the incidence and spread of PepMV to other regions of the country.

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KIAULIAUOGĖS MOZAIKOS VIRUSO (*PEPINO MOSAIC VIRUS*) DVIEJŲ SKIRTINGŲ GENOTIPŲ APTIKTŲ POMIDORUOSE LIETUVOJE CHARAKTERISTIKA

Marija Žižytė, Donatas Šneideris, Irena Zitikaitė, Laima Urbanavičienė, Juozas Staniulis

Santrauka

Du kiauliauogės mozaikos viruso (*Pepino mosaic virus*, PepMV) izoliatai buvo aptikti Lietuvos gamybiniuose šiltnamiuose auginamuose pomidoruose (*Lycopersicon esculentum* Mill.). Sekoskaitos metodu gautos PepMV izoliatų apvalkalo baltymo geno sekos buvo naudojamos filogenetinei analizei. Gautos nukleotidų sekos buvo palygintos tarpusavyje bei su kitomis PepMV izoliatų atitinkamomis sekomis, gautomis iš tarptautinės genų banko duomenų bazės. Apvalkalo baltymo geno sekos analizė parodė, kad abiejų Lietuvoje aptiktų PepMV izoliatų apvalkalo baltymo geno sekos panašumas yra 78,3%. Šie izoliatai buvo priskirti dviems skirtingiems EU ir CH2 PepMV genotipams. Šiuo metu nėra kiauliauogės mozaikos virusui atsparių pomidorų veislių, todėl svarbu nustatyti PepMV išplitimo laipsnį siekiant užkirsti kelią tolimesniam jo plitimui Lietuvoje.