Acta virologica **58**: 364 – 367, 2014 doi:10.4149/av_2014_04_364

Analysis of the complete sequences of two biologically distinct Zucchini yellow mosaic virus isolates further evidences the involvement of a single amino acid in the virus pathogenicity

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Received July 2, 2014; accepted November 10, 2014

Summary. – The complete genome sequences of two Slovak Zucchini yellow mosaic virus isolates (ZYMV-H and ZYMV-SE04T) were determined. These isolates differ significantly in their pathogenicity, producing either severe or very mild symptoms on susceptible cucurbit hosts. The viral genome of both isolates consisted of 9593 nucleotides in size, and contained an open reading frame encoding a single polyprotein of 3080 amino acids. Despite their different biological properties, an extremely high nucleotide identity could be noted (99.8%), resulting in differences of only 5 aa, located in the HC-Pro, P3, and NIb, respectively. *In silico* analysis including 5 additional fully-sequenced and phylogenetically closely-related isolates known to induce different symptoms in cucurbits was performed. This suggested that the key single mutation responsible for virus pathogenicity is likely located in the N-terminal part of P3, adjacent to the PIPO.

Keywords: ZYMV; genome; pathogenicity; P3 protein; PIPO

The presence of ZYMV (the family *Potyviridae*, the genus *Potyvirus*) in former Czechoslovakia had been reported since 1991 (Chod and Jokes, 1991). The genome of ZYMV, like other potyviruses, consists of an approximately 9.6-kb positive-sense, single-stranded RNA molecule that contains one open reading frame (ORF) encoding a large polyprotein precursor. It is cleaved into ten mature multifunctional polypeptides by three virus-encoded proteinases (Urcuqui-Inchima *et al.*, 2001). The presence of an additional small open reading frame in the +2 frame relative to the P3 (termed *pipo*) was recently discovered. *Pipo* is characterized by a highly conserved $G_{1\text{-}2}A_{6\text{-}7}$ motif at the beginning of its ORF. *Pipo* encodes a ~7 kDa peptide, which is expressed as a 25 kDa fusion protein with the N-terminal part of P3 via ribosomal frameshifting or transcriptional slippage. This polypeptide has been called P3N-PIPO (Chung *et al.*, 2008).

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Abbrevations: DAS-ELISA = double antibody sandwich ELISA; dpi = days post infection; *pipo* = pretty interesting *Potyviridae* ORF; ZYMV = Zucchini yellow mosaic virus

Most cultivated cucurbit species have a known resistance or tolerance to ZYMV (Desbiez and Lecoq, 1997). Genetic determinants of plant virus-mediated virulence are usually located on viral encoded proteins, and might involve one or more amino acids. In the case of ZYMV, the HC-Pro and P3 proteins are considered as key virulence factors (Desbiez *et al.*, 2003; Gal-On, 2007). In this work, we report the complete nucleotide sequences of two isolates different in their pathogenicity: aggressive (ZYMV-H) and mild (ZYMV-SE04T); additionally, we compare their amino acid sequences with those of other ZYMV isolates available in GenBank in order to highlight possible amino acid substitution(s) linked to the virus pathogenicity.

Both of the ZYMV isolates used (SE04T and H), which had originated from naturally infected squash (*Cucurbita pepo*), had been partially characterized previously (Glasa *et al.*, 2007). The viruses were maintained long-term at -80°C, prior to propagation in the natural host *C. pepo*, cv. Zelena under growth chamber conditions (14 hr photoperiod, 50 μE/m^{2}s exposure, day/night temperature: 25/18°C). Total

RNAs were extracted from *C. pepo* cv. Zelena at 21 days after inoculation and used for the isolation by a NucleoSpin RNA Plant kit (Macherey-Nagel, Duren, Germany) according to the manufacturer´s manual. The total RNAs were used as the template for the amplification of various genomic regions by the two-step RT-PCR protocol. Several pairs of primers were designed according to the nucleotide sequences of ZYMV-Kuchyna (Acc. No. DQ124239) in order to amplify the overlapping PCR fragments covering the whole ZYMV genome (primer sequences are available from the authors). A primer corresponding to the first 18 nt of the 5´UTR was used as the 5´primer for cDNA amplification of this part of the genome. All PCR fragments were generated using the proofreading TaKaRa Ex Taq™ polymerase (TakaraBio, Inc.) under the following conditions: 5 min initial denaturation (94°C), 35 cycles at 94°C for 1 min, 54°C for 45 sec, 72°C for 1 min to 1.5 min, and 10 min final extension (72°C). The PCR products were gel-purified (Wizard®SV Gel and PCR Clean-Up System, Promega) and directly sequenced (Bitcet, Bratislava) using the same oligonucleotides as had been used for the PCR.

Sequence analyses were performed using MEGA5 (Tamura *et al*., 2011). Complete sequences were compared with the sequences available in the GenBank database (www.ncbi.nlm.nih.gov). The phylogenetic tree was inferred, using the neighbor-joining algorithm implemented in MEGA5. The coding sequences were translated into amino acid sequences using the Expasy translate on-line tool (http://web.expasy.org/translate/), and aligned with the ClustalW algorithm available from EMBL-EBI (http://www.ebi.ac.uk/). The following amino acid sequences were used for phylogenetic analysis: B* (AY188994), Fars (JN183062), Kuchyna (DQ124239), 2002 (AB188116), AG (EF062583), KR-PA (AY278998), KR-PE (AY278999), KR-PS (AY279000), TW-TN3 (NC003224), CU (AJ307036), SG (AJ316228), WG (AJ316229), WM (AJ515911), DD056806, Singapore (AF014811), Reunion Island (L29569), California (L31350).

The full-length genome sequences determined for both the aggressive ZYMV-H and the mild ZYMV-SE04T isolates (Glasa *et al*., 2007) have been deposited to the GenBank database under Acc. Nos KF976712 (H) and KF976713 (SE04T). Interestingly, despite their clear-cut pathological distinctiveness, very high sequence identity (reaching 99.8% at the nt level) was observed between isolates. As reported before from partial sequences, the analyzed Slovak and Czech isolates were mutually closely related to the Slovak isolate ZYMV-Kuchyna (Glasa *et al*., 2007). A single ORF was located between nt positions 140–9382, encoding a putative polyprotein of 3080 aa for each isolate. The ZYMV-H and ZYMV-SE04T amino acid sequences obtained have been used for the generation of a phylogenetic tree along with 17 other available protein sequences (geographically and phenotypically distinct isolates) (Fig. 1). This analysis also confirmed the high amino acid sequence identity among central European ZYMV isolates.

To identify amino acid differences related to different phenotypic expressions of the isolates (i.e., symptoms in a natural host), a comparison among 3 isolates that differed in their pathogenicity was performed on ZYMV-H (aggressive),

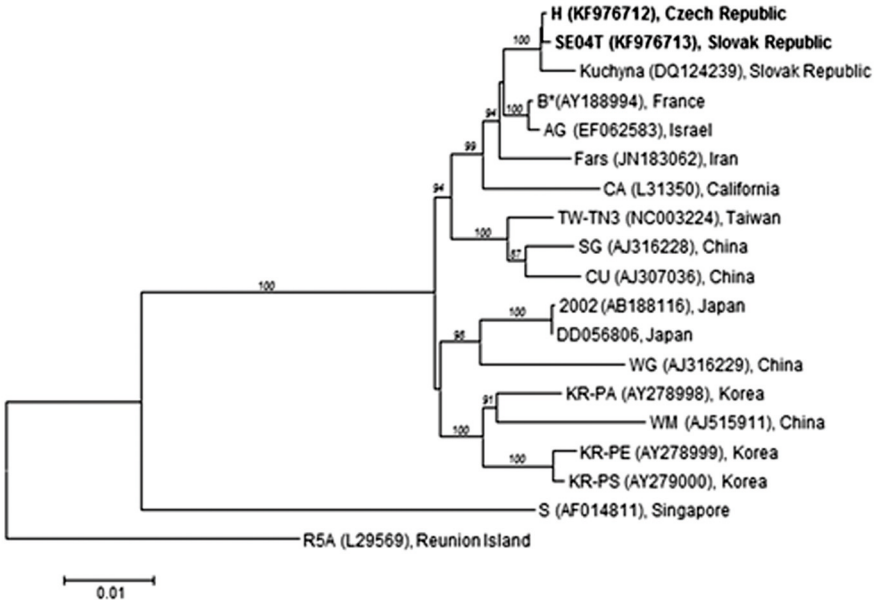

Fig. 1
Phylogenetic analysis of the complete polyprotein amino acid sequences of 19 ZYMV isolates from geographically distinct areas
The scale bar indicates a genetic distance of 0.01.

Table 1. Differences found in polyprotein amino acid sequences of seven phylogenetically related ZYMV isolates with different pathogenicity

	P1			HC-Pro			P3		CI		VpG	NIa			NIb	
aa	195	236	370	434	503	578	917	1653	1654	1720	2035	2194	2205	2516	2562	2667
Kuchyna	D	K	G	H	L	S	**R**	N	S	I	D	S	T	D	R	P
H	E	E	E	Y	L	S	**K**	T	R	M	N	L	I	D	G	S
SE04T	E	E	E	H	F	N	**R**	T	R	M	N	L	I	N	G	S
B*	E	E	E	H	L	S	**W**	T	R	M	N	L	I	D	G	S
AG	E	E	E	H	L	S	**R**	T	R	M	N	L	I	D	G	S
California	E	E	E	H	L	S	**R**	N	S	M	N	L	I	D	G	S
Fars	E	E	E	H	L	S	**N**	T	R	M	N	L	I	D	G	S

Positions have been selected on the basis of comparisons of Slovak and Czech isolates. Amino acid changes relative to the virus phenotypic behavior are indicated in bold.

ZYMV-SE04T (mild), and ZYMV-Kuchyna (moderate). Overall, 16 differences in amino acid sequences among the three isolates, and only 5 aa changes between SE04T and H, could be identified. While P3 and NIb proteins contained a single amino acid change, 3 of 5 substitutions have been identified in the HC-Pro region (Tab. 1). When we expanded our comparison to 4 additional polyprotein amino acid sequences of phylogenetically close isolates (B*, AG, California, Fars), more than 110 amino acid changes along the whole polypeptide could be identified, confirming a high variability among isolates. However, when considering only amino acid changes linked to different phenotypic behavior of the isolates, the number of mutations was reduced to one located within the P3 region at aa position 917 (Table 1). A single point mutation in the P3 protein of ZYMV, located at aa position 917, was proven to be enough to induce tolerance breaking (increased aggressiveness) on tolerant zucchini plants (Desbiez *et al*., 2003; Glasa *et al*., 2007). Several research papers showed that P3 protein may be involved in many processes including resistance breaking (Johansen *et al*., 2001; Jenner *et al*., 2002, 2003). When a putative peptide PIPO was identified, key studies reported that P3N-PIPO plays a central role as a movement protein and a virulence factor (Vijayapalani *et al*., 2012). It is still unclear whether the virulence determinant is P3 or P3N-PIPO. Wen

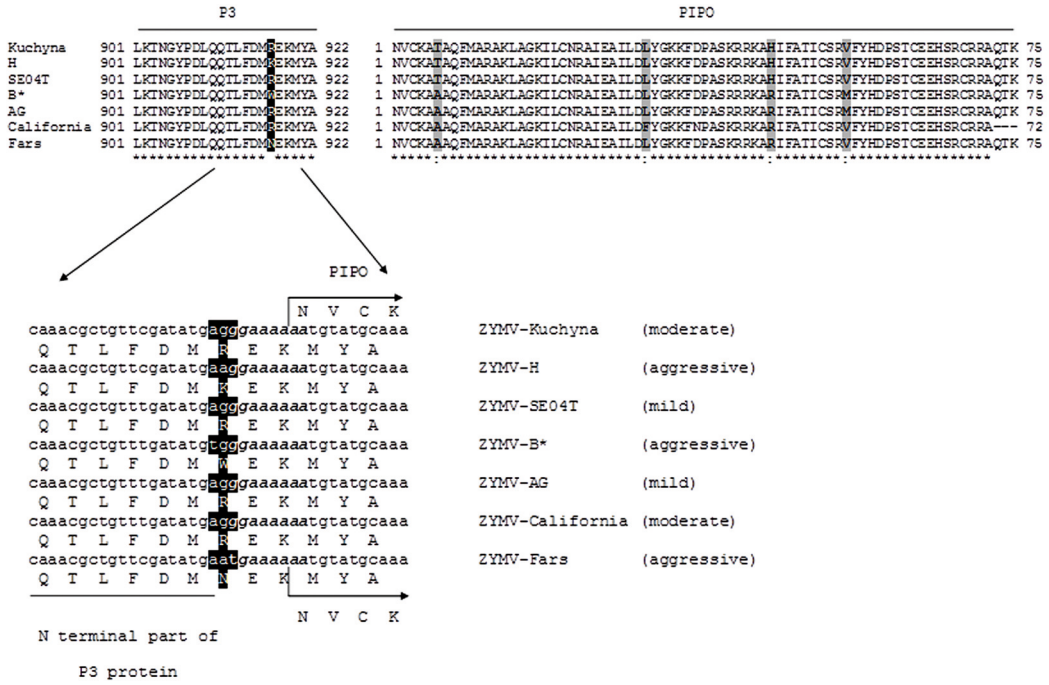

Fig. 2

Localization of R_{917} mutation site within P3N-PIPO protein and comparison of the putative PIPO peptides of seven phylogenetically close isolates different in their pathogenicity

R_{917} mutation site is black boxed. The arrows indicate the start of the putative PIPO, and the $G_{1\text{-}2}A_6$ *pipo* conserved motif is shown in italics. Amino acid differences in PIPO peptides are indicated in gray boxes.

and Hajimorad (2010) reported that mutation of the putative SMV (*Soybean mosaic virus*) PIPO inhibits cell-to-cell movement, but does not affect the virulence. This effect was the result of a mutation in the P3 cistron upstream of PIPO (Wen *et al.*, 2011). Our results presented here support the previous observation that a single amino acid mutation at the position 917 affects the virus pathogenicity. This aa is located in the N-terminal part of P3, but very close to the beginning of the putative PIPO (Fig. 2). Recently, P3N-PIPO has been shown to be a virulence determinant that allows infection of plants carrying recessive resistance alleles (Choi *et al.*, 2013).

To locate the aa differences between isolates, we also analyzed the putative PIPO peptides of 7 phylogenetically close isolates (H, SE04T, Kuchyna, Fars, B*, AG, California), which have different pathogenicity on cucurbits. The highly conserved G_2A_6 motif at the beginning of *pipo* is located at nt positions 2890–2897 in all of these isolates except for ZYMV-Fars. ZYMV-Fars *pipo* contains an in-frame stop codon (UGA) at nucleotide positions 2890–2892. The $G_{1\text{-}2}A_{6\text{-}7}$ motif begins with the *pipo*-frame stop codon UGA instead of GGA in 16 of 48 RefSeqs (Reference Sequences) (Chung *et al.*, 2008). The last two ´A´s from the (G) GAA_AAA_A frame have been annotated as the first 2 ´A´ of the putative PIPO ORF. It is expected that GAA and AAA are decoded by the ribosome in the polyprotein frame before the slippage (-1 or +2) occurs (Dr. A. Firth, personal communication). However, we have detected 4 aa differences in the PIPO among the isolates, although none of them was connected with the phenotypic behavior of the respective isolate (Fig. 2).

The analysis of aa differences between the aggressive and mild ZYMV isolates further confirms the key role of the P3N cistron in the virus pathogenicity.

Acknowledgements. This work was supported by the grant VEGA 2/0156/12 from the Scientific Grant Agency of the Ministry of Education and Slovak Academy of Sciences. J.S. receives the support from the Ministry of Agriculture, Czech Republic (project MZe 0002700604). Authors thank Dr. Andrew Firth for a fruitful discussion.

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