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Sequence variability of helper component protein of potato virus Y identified by thermodynamic methods

J. ŠKOPEK*,** and J. MATOUŠEK*,[1]

*Institute of Plant Molecular Biology, Academy of Sciences of the Czech Republic, Branišovská 31, CZ-37005 České Budějovice, Czech Republic**
*Faculty of Biological Sciences, University of South Bohemia, Branišovská 31, CZ-37005, České Budějovice, Czech Republic***

Abstract

An extent of helper component protein (HC-Pro) sequence variability within virus population of single Czech isolate of potato virus Y^{NTN} (PVY^{NTN}) Nicola was identified by temperature-gradient gel electrophoresis (TGGE) and heteroduplex analysis. HC-Pro region was approximated with 6 pairs of primers derived from Hungarian PVY^{NTN} isolate (sequence AC M95491). Immunocapture reverse transcription - polymerase chain reaction (RT-PCR) was used to obtain six mixtures of individual overlapping polymerase chain reaction (PCR) products. cDNA libraries were prepared by cloning of purified PCR products in pCR-Script vector and screened by heteroduplex analyses. 15 different subfragments within the HC-Pro region were isolated and sequenced. In comparison to AC M95491, 19 nucleotide changes were identified, 13 led to amino acid (aa) changes. In comparison to available post-transcriptional gene silencing (PTGS) specific suppressor HC-Pro sequences of potyviruses we found out 4 aa changes in conserved regions.

Additional key words: cDNA analysis, heteroduplex analysis, PTGS suppressor, TGGE.

Introduction

The suppression of RNA silencing by plant viruses represents a viral adaptation to a host antiviral defence. Upon activation by double-stranded RNA species, RNA silencing mechanism can degrade viral and transgene RNAs as well as endogenous mRNAs in a sequence-specific manner. RNA silencing occurs in a variety of eukaryotic organisms and has been described as post-transcriptional gene silencing (PTGS) and RNA-mediated virus resistance (RMVR) in plants, quelling in *Neurospora* and RNA interference (RNAi) in *Caenorhabditis elegans* and *Drosophila* (for reviews see Li and Ding 2001, Vance and Vaucheret 2001). Despite of the identification of several viral PTGS suppressors, their structural features and functional domains, as well as the mechanism of their interaction(s) with the host metabolism, remain to be elucidated.

One of the most important plant viruses encoding PTGS suppressor-HC-Pro is potato virus Y (PVY). The monopartite genome of PVY consists of about 9700 nt positive-sense RNA with virus protein genome-linked (VPg) covalently linked to its 5'-end and a poly (A) tail at the 3'-end. Single open reading frame is translated to a polyprotein, which is autoproteolytically processed into 10 proteins: P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-proteinase, NIb and CP (Shukla *et al.* 1994). Among these proteins, HC-Pro is a protein with multifunctional

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Abbreviations: aa - amino acid; HC-Pro - helper component protein; PCR - polymerase chain reaction; PVY - potato virus Y; RFLP - restriction fragment length polymorphism; RMVR - RNA-mediated virus resistance; RNAi - RNA interference; RT-PCR - reverse transcription - polymerase chain reaction; TEV - tobacco etch virus; TGGE - temperature-gradient gel electrophoresis; ZYMV - zucchini yellow mosaic virus.
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[1] Corresponding author; fax: (+420) 385310356, e-mail: jmat@umbr.cas.cz

activities. Besides the suppressor function, where it targets a maintenance step of the RNA silencing pathway (Li and Ding 2001), HC-Pro participates in the potyvirus replication cycle, aphid-mediated transmission, polyprotein processing and long-distance virus movement (Atreya *et al.* 1992, Llave *et al.* 2000). Due to unsuccessful efforts of cloning PVY cDNA in *Escherichia coli* (Sasaya *et al.* 2000), most information about HC-Pro molecular functions has been deduced from mutational analysis of other potyviruses. The biologically active form of HC-Pro is speculated to be a dimer (Shukla *et al.* 1994). This is supported by a finding of Urcuqui-Inchima *et al.* (1999), who demonstrated that amino acid changes in His^{23}, Cys^{25} and Cys^{53} to Gly of PVY HC-Pro had a dramatic impact on efficiency of self-interaction. HC-Pro as protease is responsible for HC-Pro-P3 cleavage; this activity is determined in the C-terminal half of HC protein. Site-directed mutagenesis of tobacco etch virus (TEV) HC-Pro showed a crucial role of Cys (114 amino acid upstream the C-terminal Gly) and His (41 amino acid upstream the C-terminal Gly) for proteolytic activity, positions -4 (Tyr^{453}), -2 (Val^{455}), -1 (Gly^{456}) and +1 ($Gly^{1(P3)}$) were found to be critical, while positions -5, -3 and +2 not (Shukla *et al.* 1994). As aphid transmission factor, HC protein is able to interact collaterally with the virus particle and the vector aphid stylet (Shukla *et al.* 1994). On the basis of HC-Pro amino acid region comparisons between aphid transmitted and defective strains of PVY and other potyviruses, Lys of the LysIleThrCys motif is thought to be important in interactions with aphid stylets (Blanc *et al.* 1998), nevertheless additional differences have been noted in other N-terminal amino acids that correlate with defective HC-Pro in PVY (Thornbury *et al.* 1990, Canto *et al.* 1995, Blanc *et al.* 1998). A replacement of proline by alanine in the conserved ProThrLys in HC-Pro region of zucchini yellow mosaic virus (ZYMV) resulted in loss of virion-binding ability of ZYMV (Peng *et al.* 1998). The presence of a highly conserved arrangement of cysteines and histidines in N-terminal part, which is similar to the consensus sequence of metal-binding sites in nucleic acid binding proteins, is characteristic for potyviruses (Shukla *et al.* 1994). Based on experiments with TEV HC-Pro it is supposed that the role of HC-Pro in long-distance movement and maintenance of genome amplification is an indirect consequence of the role of HC-Pro as a suppressor of posttranscriptional gene silencing (Kasschau *et al.* 2001).

During the last decades besides traditional strains of PVY described as PVY^{O}, PVY^{N} and PVY^{C}, there were found new unusual isolates or pathotypes of PVY described as PVY^{NTN}, PVY-Wi, PVY^{Z} and PVY^{ZE} (Brunt *et al.* 2001). In addition, it has been found that most of isolates of this virus contain minor sequence variants or quasispecies (Matoušek *et al.* 2001). Although the HC-Pro region of PVY has been included in restriction fragment length polymorphism (RFLP) analyses (Glais *et al.* 1998, 2002), a complete extent of HC-Pro variability is not known, because of significant limitations of this method. However, these data would be important to understand a proteomics of HC-Pro and its sequence variability in relation to general fitness of virus quasispecies (Eigen 1993), as well as for the understanding the mechanisms of virus evolution and adaptation to the new hosts. Moreover, HC-Pro and other PTGS suppressors are now widely used in plant biotechnology to investigate function of host genes and transgenes (Wang and Waterhouse 2001).

In the present study we analysed an extent of HC-Pro sequence variability by TGGE and heteroduplex analyses. Both these methods provide a fine and effective screening for minor sequence variants and sequence mixtures (Matoušek *et al.* 2000a,b). Using these methods, we selected, sequenced and characterised subsequence variants of HC-Pro within single isolate PVY^{NTN} Nicola of the Czech origin.

Materials and methods

PVY isolate and its biological and serological features: Isolate PVY^{NTN} Nicola originating from the Czech Republic was those characterized previously by Ptáček *et al.* (2002) using a combination of biological, serological and molecular biology methods. This isolate represents a typical PVY^{NTN} strain showing selective reaction to PVY-N antibody, typical veinal necroses on tobacco Samsun indicator plants and having ability to induce characteristic PTNRD symptoms as described by Beczner *et al.* (1984) for classical Hungarian PVY^{NTN} isolate.

DNA primers and immunocapture RT-PCR: For RT-PCR, the RT and PCR primers were derived from the sequence of a Hungarian isolate of PVY^{NTN} (EMBL AC M95491). Six primer pairs derived from HC-Pro region were applied to approximating HC-Pro region by 6 overlapping fragments ranging from 220 to 425 bp (Ptáček *et al.* 2002). The thermodynamic properties and structural features of individual primer pairs were calculated using *Gene Runner version 3.02* (*Hastings Software, Inc.*, Hastings on Hudson, NY, USA). The individual primers were derived from the following positions (the optimal annealing temperatures are given after the slashes):

2 – I PCR 1121-1140 (5'CAGGCATACCAGTTGAAGAC3')
RT 1370-1351 (5'CTCAGATCAACCGGTTCAGT3') / 53 °C
2 – II PCR 1293-1310 (5'GGCAGACAAAGATCGCTT3')
RT 1588-1607 (5'CAATTTGCTTTGGCAGATAG3') / 53 °C
2 – III PCR 1554-1574 (5'CAAGAAAGGAGACATCTCGTT3')
RT 1978-1961 (5'GGTGCTTCTTAGTTGGCG3') / 53 °C
3 – I PCR 1881-1900 (5'CTCGAAGGATGGAAACTACG3')
RT 2276-2257 (5'TGCGTTTCGTGATCGACTAG3') / 55 °C
3 – II PCR 2101-2122 (5'ATTAGTGAGGAAGATGCAAAGG3')
RT 2398-2376 (5'GTGCTTAATGTCAGACTCCAAC3') / 55 °C
3 – III PCR 2329-2349 (5'TTGAAAGCATCTAGCGTGTCC3')
RT 2773-2752 (5'CAAGGAGATCTGTAGCTGCAGT3') / 55 °C

Immunocapture on ELISA plates was used for template preparation. First-strand cDNAs were synthesised with random hexanucleotides in ELISA microplates. For PCR, 0.005 cm^3 of the reverse transcriptase reaction mixture (cDNA) was amplified in a total volume of 0.05 cm^3 using the Enhanced Avian RT-PCR Kit (*Sigma-Aldrich*, Prague, Czech Republic) and the corresponding primers.

Cloning: The obtained PCR products were purified by use of the *QIAquick Gel Extraction Kit* (*Qiagen*, Hilden, Germany) and cloned into the pCR-Script™ Amp SK (+) vector using the PCR-Script™ Cloning Kits (*Stratagene*, Amsterdam, Netherlands). Selected cDNA libraries contained 193, 32, 208, 20, 38 and 27 positive clones containing fragments 2-I, 2-II, 2-III, 3-I, 3-II and 3-III of PVY^{NTN} Nicola, respectively. These clones were verified by molecular hybridization using purified PCR products 2-I, 2-II, 2-III, 3-I, 3-II and 3-III as probes.

TGGE, DNA heteroduplex analysis and nucleotide sequencing: TGGE was performed in 5 % acrylamide gels using a minigel apparatus (*Biometra*, Göttingen, Germany). The gels contained acrylamide and bis-acrylamide in the ratio of 19:1 (m/m), 17.8 mM Tris, 17.8 mM boric acid, 0.2 mM EDTA pH 8.3, 0.1% TEMED, 7 M urea, 2 % glycerol and 0.06 % ammonium persulfate. An individual linear temperature gradient, 150 V, 0.5 A and 30 W were used. After electrophoresis, the gels were silver-stained according to method of Schumacher *et al.* (1986).

DNA heteroduplex analysis was performed essentially as described by Matoušek *et al.* (2001). PCR fragments were first denatured and then annealed. The annealing mixture contained 0.0058 cm^3 of a tested DNA sample (PCR product from tested bacterial clone), 0.005 cm^3 of the standard DNA (PCR from the standard bacterial clone) and 0.0012 cm^3 of 10 mM sodium cacodylate buffer pH 6.8 (1 M NaCl and 10 mM EDTA). The mixture was heated to 95 °C in a heat block and cooled in an insulation box to 40 °C in 2 h. An aliquot (0.012 cm^3) of the annealing mixture was mixed with an equal volume of a dye solution and electrophoresed at selected temperatures (see results). Acrylamide gels had the same composition as those used in TGGE. After electrophoresis, the gels were silver-stained as above.

Sequencing was performed using an automated sequencer *ALF II* (*Amersham Biosciences*, Uppsala, Sweden) using the *Thermo Sequenase* Dye Terminator Cycle Sequencing Kit* (*Amersham Biosciences*). Sequence comparisons were performed using the computer programme *Omiga*™ 2.0 (*Oxford Molecular Ltd.*, London, UK) and *Vector NTI Suite 5.5* (*Informax, Inc.*, Oxford, UK). For comparison, we used the sequence of Hungarian isolate PVY^{NTN} deposit at the NCBI database (http://www.ncbi.nlm.nih.gov/) under AC M95491. Nucleotide and amino acid changes are numbered in this study with respect to HC-Pro gene of Hungarian isolate (287.-742. aa in the polyprotein).

Results and discussion

We aimed to perform a detailed analysis of HC-Pro variation within PVY population of single virus isolate. For this reason we selected PVY^{NTN} isolate Nicola for which characteristic immunocapture RT-PCR products were obtained previously (Ptáček *et al.* 2002), using primer pairs for differentiating PVY^{NTN} (Glais *et al.* 1996, Weidemann and Maiss 1996, Weilguny and Singh 1998). These results together with the characteristic spectrum of immunocapture RT-PCR probing system using a set of 24 pairs of primers derived from eight regions of the PVY genome (Ptáček *et al.* 2002) suggest that this isolate did not represent a mixture of biological strains of PVY. Despite these unambiguous characteristics, it has been found previously by the use of thermodynamic methods (Matoušek *et al.* 2001) that PVY^{NTN} Nicola contained some minor sequence variants showing differences in P1

region. In our present experiments the TGGE analyses of HC-Pro region of PVY^{NTN} Nicola also showed some minor variants (not shown), confirming the presence of more complex population presumably containing quasi-species as defined by Eigen (1993). As almost neutral sequences having lower fitness, these minor variants could accumulate specific mutations, which we aimed to pre-select using TGGE and DNA-heteroduplexes methods to analyse an extent of HC-Pro variability. For these reasons six groups of subfragments overlapping HC-Pro region (Ptáček *et al.* 2002) designated 2-I, II, III and 3-I, II, III were prepared from PVY genome using immunocapture RT-PCR and cloned in pCR-Script vector. From TGGE profiles of individual subfragments (Fig. 1) the individual melting points were deduced and used for library screening using DNA heteroduplexes method as described by Matoušek *et al.* (2000a) (Table 1, Fig. 2). Also these results confirmed that no single HC-Pro sequences were presented in isolate Nicola and instead, a population of sequence was detected. In total, 15 different sequence subfragments of HC-Pro were selected from individual cDNA libraries by means of heteroduplex analyses (Figs. 2, 3, Tables 1, 2). Subfragments 2-I-4, 2-III-50, 3-II-2 and 3-III-2 appeared as the dominant forms by heteroduplex analysis (not shown). On the other hand, it was not possible to distinguish, whether subfragments 2-II-31, 2-II-31, 3-I-9 and 3-I-10 form dominant or minor forms by heteroduplex analysis.

In order to deduce a character of individual mutations these clones were sequenced and aligned (Fig. 3). In comparison to Hungarian PVY^{NTN} isolate (AC M95491) 19 nucleotide changes were identified, 13 led to aa changes, one to nonsense mutation (Fig. 3, Table 2). 7 nucleotide changes represented the change in the first triplet position, 5 and 7 in the second and third triplet positions, respectively. 53 % of nucleotide changes represent Pu->Pu changes, 32 % Py->Pu and 16 % Py->Py. Molecular variability in the HC-Pro region of PVY^{NTN} isolate Nicola was found at amino acid positions 47 (Pro-Ala), 61 (Ala-Thr), 163 (Phe-Tyr), 169 (Asp-Glu), 181 (Asn-Tyr), 190 (Asn-Ser), 197 (Asn-Ser), 241 (Asn-Asp), 364 (Lys-Arg), 372 (Pro-Ala), 424 (Thr-Ala) and 441 (Phe-Ser). Amino acid change Val^{336} was found to be present in both analysed variants of the Czech isolate PVY^{NTN} Nicola, while Ile^{336} was present in

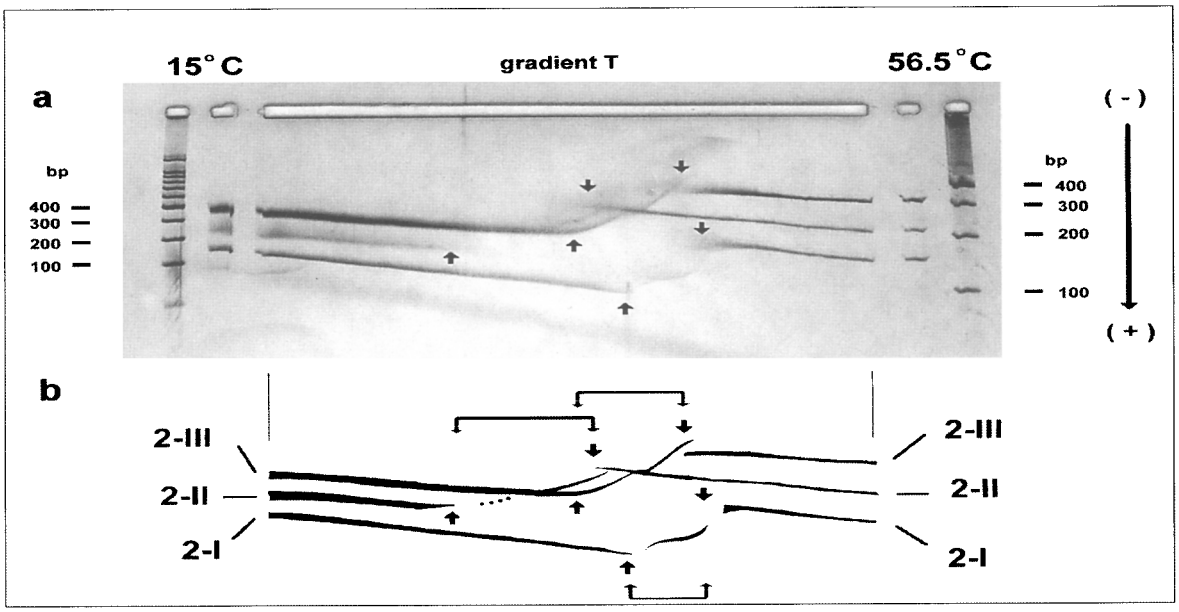

Fig. 1. An example of analysis of subfragments 2-I-4, 2-II-31 and 2-III-4 by TGGE (*a* - the TGGE profiles in acrylamide gel; *b* - the schematic drawing of TGGE profiles above; the transition curves are indicated by *arrows*).

Table 1. Features of analysed fragments 2-I, 2-II, 2-III, 3-I, 3-II and 3-III. Melting points were derived from TGGE profiles.

Fragment	2-I	2-II	2-III	3-I	3-II	3-III
Fragment size [bp]	250	315	425	220	298	392
Melting point [°C]	40.6	37.6	38.6	40.5	40.9	40.1
Number of sequence variants	3	2	2	2	4	2

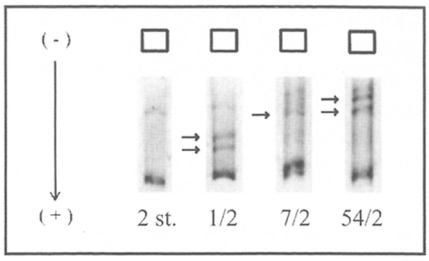

Fig. 2. An example of analysis of HC-Pro library by the method of pre-formed DNA heteroduplexes. Phenolised cDNA fragments 3-II were hybridised to tester cDNA of the clone No. 2 and electrophoresed at constant temperature of 40.9 °C. Then the gel was stained with silver nitrate. The positions of the retarded heteroduplexes corresponding to samples 1/2, 7/2 and 54/2 are indicated by *arrows*.

the same position in Hungarian PVY^{NTN} isolate (Fig. 3), suggesting that this difference could be specific for the Czech isolate.

Having identified HC-Pro variable amino acids, we extended our analyses to phylogenetic comparisons. Thornbury *et al.* (1990) published conserved HC-Pro amino acids of six Potyvirus isolates available (PVC, PVY, PVYn, PPV, TEV, TVMV). Nowadays, 58 HC-Pro sequences of potyviruses transmitted by aphids (Walkey 1991) is presented in the database of NCBI (http://www.ncbi.nlm.nih.gov/) these new sequences we included in our comparisons (Table 3) to find out the conserved motifs characteristic for potyviruses (marked in Fig. 3). These motifs we compared with PVY-specific HC-Pro sequences obtained experimentally. It follows from our comparisons that aa variation of HC-Pro within analysed virus population included four conserved motifs:

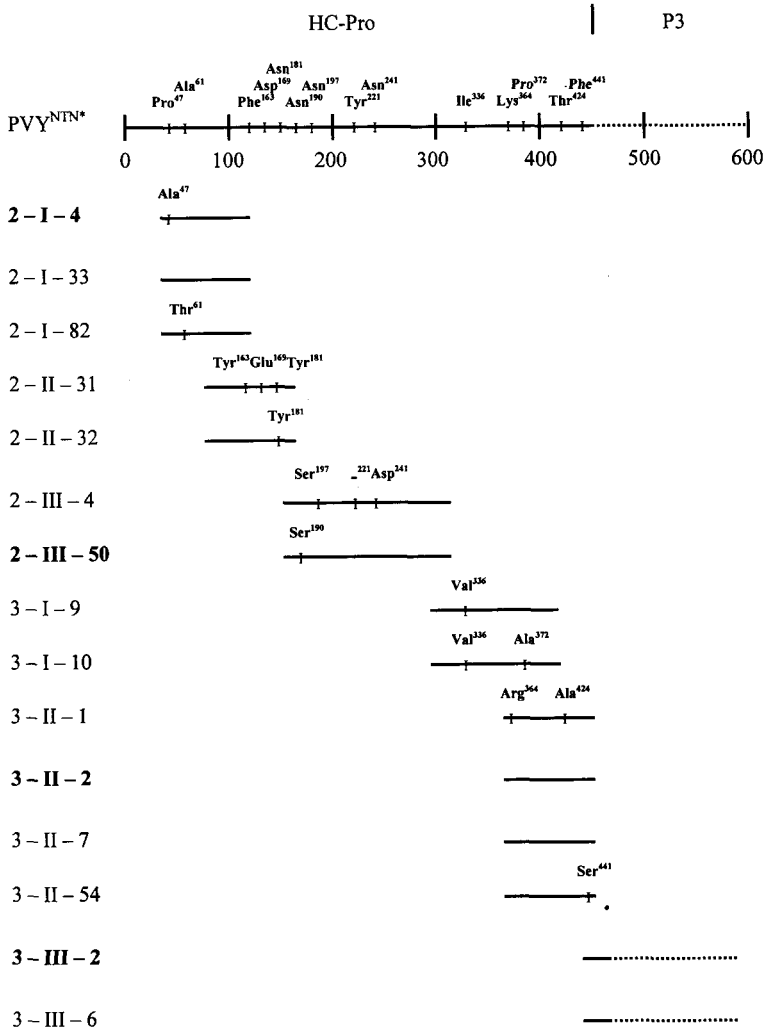

Fig. 3. Multiple sequence alignment at amino acid level of 15 cloned subfragments of HC-Pro regions 2-I, 2-II, 2-III, 3-I, 3-II and 3-III of Czech isolate PVY^{NTN} Nicola. PVY^{NTN*} - represents the sequence of Hungarian isolate (AC M95491). The sequence variants are designated as in Table 2. Sequences which appear as dominant forms are indicated strengthenly. HC-Pro/P3 protein boundary is designated. P3 region is marked by a *dashed line*. Subfragments 3-III-2 and 3-III-6 did not vary in HC-Pro region, some differences were found in P3 region (not shown).

Table 2. Analysis of nucleotide and amino acid differences within individual subfragments in comparison to Hungarian PVY^{NTN} isolate (AC M95491) (* - positions of nucleotide changes are given after the slashes; ** - sequences which appear as dominant forms are indicated strengthenly; *** - subfragments 3-III-2 and 3-III-6 do not vary in examined HC-Pro region but in P3 region (not shown).

Sequence variant	"Silent" changes No.	position	Missense changes No.	position	Nonsence changes No.	position	Total changes No.
2-I-4**	1	A/ 126 -> G*	1	C/ 139 -> G*	0		2
2-I-33	0		0		0		0
2-I-82	0		1	G/ 181 -> A*	0		1
2-II-31	0		3	T/ 488 -> A* T/ 507 -> A* A/ 541 -> T*	0		3
2-II-32	0		1	A/ 541 -> T*	0		1
2-III-4	1	C/ 702 -> T*	2	A/ 590 -> G* A/ 721 -> G*	1	T/663 -> G*	4
2-III-50**	1	C/ 702 -> T*	1	A/ 569 -> G*	0		2
3-I-9	2	G/ 966 -> A* C/1011 -> *	1	A/1006 -> G*	0		3
3-I-10	2		2	A/1006 -> G* C/1114 -> G*	0		4
3-II-1	0		2	A/1091 -> G* A/1270 -> G*	0		2
3-II-2**	0		0		0		0
3-II-7	1	G/1278 -> A*	0		0		1
3-II-54	0		1	T/1322 -> C*	0		1
3-II-2**,***	0		0		0		0
3-III-6***	0		0		0		0

Table 3. The list of HC-Pro sequences of aphid-transmitted potyviruses (Walkey 1991) used for comparison.

Virus	HC-Pro sequences
Bean common mosaic virus	AC AJ312438, AC NC003397, AC U19287
Bean yellow mosaic virus	AC D 83749
Clover yellow vein virus	AC AB011819
Cowpea aphid-borne mosaic virus	AC AF348210
Dasheen mosaic virus	AC AJ298033
Japanese yam mosaic virus	AC NC000947, AC AB016500
Papaya ringspot virus	AC NC001785, AC NC002814, AC X97251
Pea seed-borne mosaic virus	AC AJ252242, AC NC001671, AC X89997
Peanut mottle virus	AC NC002600
Pepper mottle virus	AC NC001517
Plum pox virus	AC AJ243957, AC M92280, AC NC001445, AC X81803, AC X16415
Potato virus A	AC AJ131400, AC AJ131401, AC AJ 131402, AC AJ131403, AC AJ296311, AC NC001649
Potato virus V	AC AJ43766
Potato virus Y	AC AF166115, AC Z50041, AC Z50042, AC Z50043, AC M38377, AC M37180, AC U09509, AC X12456, AC M95491, AC X97895
Soybean mosaic virus	AC AF241939, AC NC002634
Sugarcane mosaic virus	AC AJ310102, AC AJ310103, AC AJ310104, AC AJ310105, AC NC003398
Tobacco etch virus	AC L38714, AC M11458
Tobacco vein mottling virus	AC U38621, AN NC001768
Turnip mosaic virus	AC AF394601, AC AF394602, AC D83184, AC NC002509
Yam mosaic virus	AC D42596
Zucchini yellow mosaic virus	AC NC003224, AC L29569, AC AF014811

in positions 181 (Asn-Tyr) in $Phe^{179}Arg^{180}Asn^{181}Lys^{182}$ motif, 197 (Asn-Ser) in $Cys^{195}Asp^{196}Asn^{197}Gln^{198}Leu^{199}Asp^{200}$ motif, 364 (Lys-Arg) in $Thr^{363}Lys^{364}$ motif and 441 (Phe-Ser) in position Phe^{441}. On the other hand, no changes were found in conserved aa positions $Lys^{50}Ile^{51}Thr^{52}Cys^{53}$, $Pro^{308}Thr^{309}Lys^{310}$, Cys^{342}, His^{415}, Tyr^{453}, Val^{455}, Gly^{456} and $Gly^{1(P3)}$. Because there is not any detailed information available about possible functional features of the conserved regions, where aa changes occurred, we can not decide whether or not the observed changes lead to some functional changes, but such possibility is quite probable. For instance, aa pairs Asn/Tyr and Asn/Ser are considered to be non homologous. Especially in position 181 small polar and turnlike Asn is changed by hydrophobic Tyr, which could influence the structure of this protein domain. Furthermore, three of the changes in conserved regions 197 (Asn-Ser) in $Cys^{195}Asp^{196}Asn^{197}Gln^{198}Leu^{199}Asp^{200}$ motif, 364 (Lys-Arg) in $Thr^{363}Lys^{364}$ motif and 441 (Phe-Ser) in Phe^{441} occurred in minor population variants, presumably having lower fitness. On the other hand, we did not find out any sequential aa change in the regions marked by others, like in $Lys^{50}Ile^{51}Thr^{52}Cys^{53}$ and $Pro^{308}Thr^{309}Lys^{310}$ motifs important to mediate the joint between coat protein and aphid stylets (Blanc *et al.* 1998, Peng *et al.* 1998) or in amino acid positions Cys^{342} and His^{415} important for the function of HC-Pro as protease (Shukla *et al.* 1994). Also amino acids Tyr^{453}, Val^{455}, Gly^{456} and $Gly^{1(P3)}$ important for the specificity of the restriction site between HC-Pro and P3 were unchanged within the analysed population. There was a stop codon detected in the minor sequence variant 2-III-4, suggesting non functional protein. Although we cannot decide whether this is a natural mutation or not, in this fragment several mutations were detected, which most probably appeared during the subsequent RNA replication cycles and not due to RT-PCR amplification which was mediated in our experiments by a high fidelity system.

A question arises as to the relation between an extent of sequence variability of HC-Pro and viral features involved in the interaction of particular population of virus isolate with the host genotype, as well as the appearance of minor sequence variants in the viral population. HC-Pro as suppressor of PTGS participates in regulation of the antiviral defence mechanism of the host plant presumably *via* interaction with siRNA and possibly miRNA pathways (Li and Ding 2001). Considering that the host spectra of potyviruses transmitted by aphids vary, one of the reasons of such variability may be the ability or inability of the particular isolate to suppress the defence mechanism of the particular host plant. Because of the involvement of HC-Pro in this regulation, one can assume, that particular sequential differences of HC-Pro can influence the host range, as well as the kind and severity of symptoms. It is also possible that the replication and survival of minor virus quasispecies presumably mutated in HC-Pro or in other regions are supported by a "HC-Pro efficient" form which is able to suppress efficiently plant defence mechanism. Similar synergistic effects of HC-Pro were recently described by (Yelina *et al.* 2002).

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