ANALYSIS OF VARIABILITY OF P1 GENE REGION OF N STRAIN OF POTATO VIRUS Y USING TEMPERATURE-GRADIENT GEL ELECTROPHORESIS AND DNA HETERODUPLEX ANALYSIS

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Summary. – Reaction conditions specific for reverse taranscription–polymerase chain reaction (RT-PCR) of potato virus Y strain NTN (PVY^{NTN}) were used to amplify a 394 bp fragment of the P1 gene from selected PVY isolates with the aim to study the PVY variability within this genomic region. The P1 gene fragment from the Nicola isolate (Nicola P1/1 clone) was sequenced and characterized by temperature-gradient gel electrophoresis (TGGE). The Nicola P1/1 clone differed from that from the Hungarian isolate by double point mutation resulting in two changes at the deduced amino acid level. The clone showed simple transition from double-stranded to single-stranded form with two characteristic melting end points of about 41°C and 48°C. A more complicated TGGE pattern was, however, found for the whole P1 cDNA library of the Nicola isolate, suggesting accumulation of some minor sequence variants of PVY in this isolate. Based on the TGGE pattern, 46°C was selected as the standard temperature for electrophoretic analysis of heteroduplex DNAs formed with the Nicola P1/1 DNA as reference. More than 40 other PVY isolates from PVY^N group were analysed using this method. In most cases only minor fractions of electrophoretically distinguishable DNA heteroduplexes were found, however, in most isolates of PVY^N-Wilga type, mixtures of the major sequence variants were observed. Two of these variants from the hybrid 220-5 (Czech Republic) were sequenced. Both of them differed from the Nicola P1/1 clone by 6 point mutations, which led to several changes at the amino acid level.

Key words: potato virus Y; P1 gene; Solanum tuberosum; virus quasispecies; mixed virus infection; RT-PCR; cloning; nucleotide sequencing; TGGE; heteroduplex DNA

Introduction

PVY is one of the most well-known plant viruses. It is the type member of the family *Potyviridae*. Like other potyviruses, PVY has long flexuous rod-shaped particles containing a single-stranded positive-sense RNA. Its genome of 9.7 kb (Robaglia *et al.*, 1989; Thole *et al.*, 1993; Singh and Singh, 1996) codes for a polypeptide which is proteolytically processed into 9 proteins (from the 5′- to the 3-end of the RNA: Pl, HC-pro, P3, $6K_1$, CI, $6K_2$, Nla, Nib, and CP) (Dougherty and Carrington, 1988; Robaglia *et al.*, 1989; Riechmann *et al.*, 1992). Two main biological strain groups of PVY are recognized in practice, PVY^0 and PVY^N, although within both groups, several different variants or pathotypes have been described (e.g. Glais *et al.*, 1998). For instance, PVY^N-Wilga isolates described in Poland (Chrzanowska, 1991), evoke typical vein necrosis

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Abbreviations: MAb = monoclonal antibody; 5′-NTR = 5′-non-translated region; PTNRD = potato tuber necrotic ringspot didease; PVS = potato virus S; PVY = potato virus Y; PVY^N = PVY N strain; PVY^{NTN} = PVY NTN strain; RFLP = restriction fragment length polymorphism; RT-PCR = reverse transcription–polymerase chain reaction; TGGE = temperature-gradient gel electrophoresis

on *Nicotiana tabacum* but differ from the early PVY^{N} isolates by an increased virulence for potatoes (Chrzanowska, 1994) and non-reactivity with some monoclonal antibodies to PVY^{N} (Chachulska *et al.*, 1997). Also some other isolates show mixed properties (McDonald and Singh, 1996; Glais *et al.*, 1998; Blanco-Urgoiti *et al.*, 1998) and could probably appear due to genome recombinations (Glais *et al.*,1998; Blanco-Urgoiti., 1998). The most aggressive isolates are grouped into the PVY^{NTN} subgroup, which is associated with a new potato tuber necrotic ringspot disease (PTNRD) characterized by superficial ring necrosis on potato tubers (Beczner *et al.*, 1984).

Several regions of the PVY genome, among them also the 5′-non-translated region (5′-NTR) and the P1 gene, have been analyzed for their polymorphism by restriction fragment length polymorphism (RFLP) analysis (Blanco-Urgoiti *et al.*, 1996; Glais *et al.*, 1996, 1998) or sequencing (Van der Vlugt *et al.*, 1993; Marie-Jeanne Tordo *et al.*, 1995). The PVY P1 gene region has been also selected for specific RT-PCR diagnostic procedures (e.g. Weidemann and Maiss, 1996; Weilguny and Singh, 1998) to recognize different PVY isolates. Although a significant sequence polymorphism within the PVY P1 gene region has been described in these analyses, the overal extent of PVY variability is not known, because RFLP analysis or RT-PCR methods do not reflect all nucleotide sequence variations. In addition, mixed infections with different PVY pathotypes, similar molecular forms of PVY or possible virus quasispecies (for definition see Eigen, 1993) cannot be easily recognized by these methods.

In our previous work, we characterized a remarkable sequence microvariability of potato virus S (PVS) by TGGE and sequencing methods (Matoušek *et al.*, 2000). This microvariability was mainly due to accumulation of neutral point mutations and was revealed within natural isolates of ordinary PVS. In this study, we aimed to verify the possibility whether or not the PVY variation could be detected in some PVY isolates which we characterized previously (Dědič and Ptáček, 1998) using biological, serological and RT PCR methods. It has been shown by other authors that the 5′-end of the PVY genome including the P1 gene region represents a rather variable part of this virus. If so, then it can be expected that the sequence variants which could accumulate in a population during virus replication *per se* could be more easily detectable by the analysis of this region.

In the present study, we aimed to analyse a part of the PVY P1 gene of different isolates of the PVY^{N} group by means of TGGE (Riesner *et al.*, 1989) and DNA heteroduplex analysis. These approaches, based on thermostability of selected DNA fragments, are useful for screening mixtures of virus sequence variants (Matoušek *et al.*, 2000).

Materials and Methods

PVY isolates were selected according to the results of biological, serological and RT-PCR tests described earlier (Dědič and Ptáček, 1998). Most of the PVY isolates listed in that report were also examined in this study.

RNA was isolated using the RNeasy Plant Total RNA Extraction Kit (Qiagen).

RT-PCR. The antisense primer A3 (5′-GCGTGCGATAT GTTTTTGC-3′), the sense primer S4 (5′-CAGATTGGTTCCATT GAATGC-3′) (Weilguny and Singh, 1998), and the GeneAmp RNA PCR Kit (Perkin Elmer) were employed. Pfu polymerase (Stratagene) was used for amplification of viral cDNAs under these conditions: 1 cycle at 94°C for 3 mins, 35 cycles consisting of 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min, and 1 cycle at 72°C for 10 mins.

Cloning. The obtained PCR products were purified by use of the Qiagen Gel Extraction Kit (Qiagen) and cloned into the the Script SK(+) vector using the pCR-Script Cloning Kit (Stratagene). A cDNA library was prepared from 88 clones specific for the PVY^{NTN} Nicola isolate, verified by molecular hybridization using the Nicola P1/1 clone as probe. For TGGE analysis of P1 fragment representing the whole cDNA library, PCR was performed using a mixed sample from all individual bacterial colonies composing the library.

Nucleotide sequencing was performed on an automated sequencer (ALF II, Amersham-Pharmacia) using the Thermosequenase Dye Termination Kit (Amersham-Pharmacia) with Cy5-labeled standard primers. For comparison, we used the P1 A3S4 sequence derived from the Hungarian isolate of PVY^{NTN} deposited at the EMBL database under Acc. No. M95491. Nucleotide and amino acid changes are numbered in this study with respect to the Nicola P1/1 clone as reference.

TGGE was performed in 6% acrylamide gels (140 x 140 x 1.8 mm) (Riesner *et al.*, 1989) using an apparatus produced by OMNIBIO, Brno, the Czech Republic. The gels contained acrylamide and bisacrylamide in the ratio of 19:1 (w/w), 17.8 mmol/l Tris, 17.8 mmol/l boric acid, 0.048 mmol/l EDTA pH 8.3, 0.1% TEMED, 7 mol/l urea, 2% glycerol, and 0.06% ammonium persulfate. A linear gradient of 35–57°C and 180 V were used. After electrophoresis according to method of Schumacher *et al.* (1986), the gels were silver-stained.

DNA heteroduplex analysis. DNA heteroduplexes are formed from homologous DNAs by their annealing. DNA heteroduplexes are detected by gel electrophoresis. In comparison to their native double-stranded partner DNAs, they display a retarded mobility. The annealing mixture contained 5.8 µl of a tested DNA sample (RT-PCR product), 5 µl of the [gamma-^{32}P]-ATP-labeled cDNA fragment (approx. 4 x 10^{4} cpm) from the Nicola 1 clone (reference DNA sample), and 1.2 µl of 10 mmol/l sodium cacodylate buffer pH 6.8 (1 mol/l NaCl and 10 mmol/l EDTA). The mixture was heated to 95°C in a heat block and cooled in an insulation box to 40°C in 2 hrs. An aliquot (12 µl) of the annealing mixture was mixed with an equal volume of a dye solution and electrophoresed at 46°C. Acrylamide gels had the same composition as those used in TGGE. After electrophoresis, they were subjected to autoradiography. The obtained autoradiographs were scanned using the STORM device and the ImageQuaNT software (Molecular Dynamics).

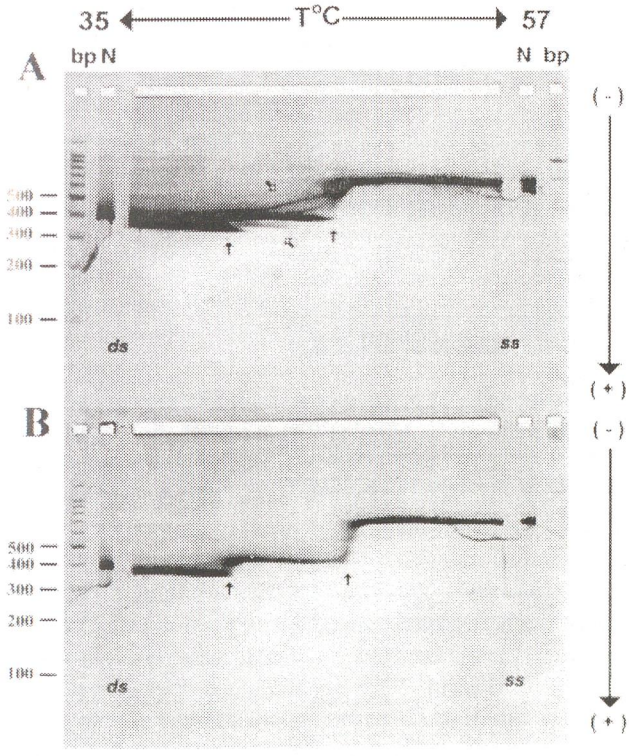

Fig. 1
Analysis of cDNA fragment of PVY P1 by TGGE
PCR fragment was prepared using primers A3 and S4 either from the cDNA library of the Nicola isolate (A) or the Nicola P1/1 clone (B) and analyzed by TGGE. The melting end-points are indicated by filled arrows and the minor cDNA structures within the transition pattern by white-hollow arrows. In the additional slots designated "bp" and "N" molecular mass markers and analyzed samples were applied, respectively. ss, ds = single-stranded and double-stranded parts of DNA patterns, respectively.

Results and Discussion

The PVY^{NTN}-specific RT PCR reaction conditions described previously by Weilguny and Singh (1998) were selected to amplify a particular 394 nt region within the P1 gene using the primers A3 and S4. In order to prepare standard cDNA for further analyses, the P1 fragment was first amplified from the Nicola isolate, which is one of the well characterized and in detail analyzed PVY^{NTN} isolates of Czech origin. A plasmid cDNA library from the Nicola isolate was established and six clones were sequenced. All of these clones were found to be identical and, therefore, the Nicola P1/1 clone was used as the reference cDNA sequence. It was found that the Nicola P1/1 clone differed from the corresponding part of PVY^{NTN} (Hungarian isolate) in 2 nucleotides (nt), nt 133 (C to A) and 341 (A to G), which caused differences in 2 amino acids. However, these amino acid pairs, valine *versus* another hydrophobic amino acid, alanine, and arginine *versus* another hydrophobic and positively charged amino acid, lysine, in the Niclola P1/1 and Hungarian isolates, respectively, have similar properties. This leads to an assumption that no principal difference in this regard exists between these two isolates. Further characteristics of the selected DNA fragments were obtained using TGGE (Fig. 1). The cloned A3S4 fragment showed simple transition from double-stranded to single-stranded DNA with two characteristic melting end-points at approx. 41°C and 48°C. A more complicated TGGE pattern and the presence of minor cDNA structures within the transition region was, however, clearly detected, when the whole cDNA library of the Nicola isolate was analyzed. These results suggest the presence of some minor fraction(s) of sequence variant(s) which differ from the predominant sequence.

One of these variants, the Nicola P1/2 clone was selected from the plasmid library and characterized. The P1/1 clone differed from the P1/2 in a single nucleotide, nt 225 (G to A). Because this mutation did not lead to any amino acid change, it could be characterized as a silent mutation. From the characterization of the Nicola isolate it can be concluded that rather minor fraction(s) of sequence variant(s) are present in this isolate. This situation differs in principle from the high extent of sequence variation which we have described for the coat protein region of the Kobra isolate of PVS using TGGE (Matoušek *et al.*, 2000).

Based on TGGE pattern, the temperature of 46°C, which is close to the second transition (Fig. 1), was selected as

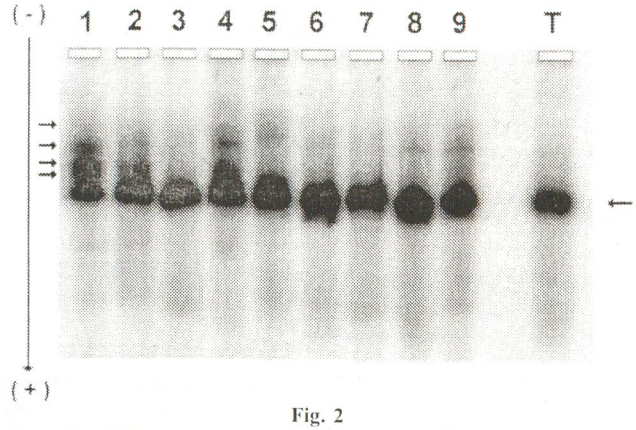

Fig. 2
Analysis of PVY variability by the method of DNA heteroduplexes
cDNA fragments were prepared from the following PVY isolates: PVY^{N}-Wilga, hybrid 220-5, Czech Republic (lane 1); PVY^{N}-Wilga, hybrid 41-97, Poland (lane 2); PVY^{NTN}, hybrid 193-2, Czech Republic (lane 3); PVY^{N}-Wilga, Nord 242, France (lane 4); PVY^{NTN}, Tu 64-8, Canada (lane 5); PVY^{NTN}, hybrid 341-3, Czech Republic (lane 6); PVY^{NTN}, Orleans, France (lane 7); PVY^{NTN}, Lukava, Czech Republic (lane 8); PVY^{NTN}, Ny, Poland (lane 9). They were hybridized to [gamma-^{32}P]-ATP-labeled reference cDNA from the Nicola P1/1 clone and electrophoresed at 46°C. Then the gel was scanned using the STORM device. The positions of the retarded heteroduplexes (lane 1) and the reference homoduplex (lane T) are indicated by arrows.

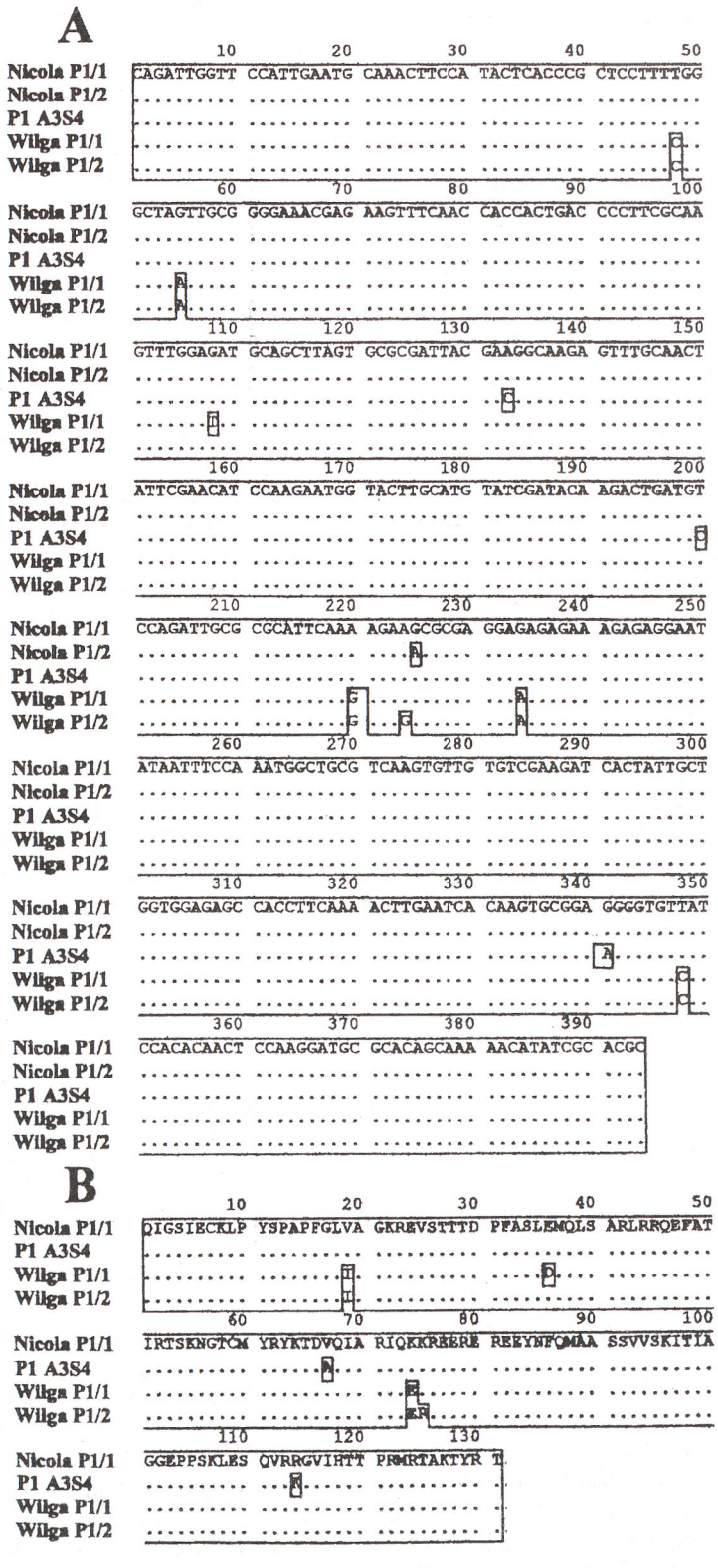

Fig. 3

Multiple alignment of cloned P1 fragments

A: two PVY^{NTN} clones(Nicola P1/1 and Nicola P1/2) and two clones of PVY^{N}-Wilga (Wilga P1/1 and Wilga P1/2) are aligned to the sequence P1 A3S4 from the Hungarian isolate of PVY^{NTN} and compared at nucleotide level. B: a comparison at amino acid level.

tandard for electrophoresis in DNA heteroduplex analysis. More than 40 isolates from the PVY^N group were subjected to DNA heteroduplex analysis and, in the majority, only minor bands of heteroduplexes were found, suggesting accumulation of minor fractions of virus sequence variants in these isolates (Fig. 2). Surprisingly, for most of the PVY^N-Wilga isolates, major bands were detected, suggesting a significant accumulation of different sequence variants in these isolates. At least two sequence variants were identified as constituents of a PVY isolate from the hybrid 220-5 (Czech Republic). Nucleic acid sequences of both clones designated Wilga P1/1 and Wilga P1/2 were compared with those of Nicola and the Hungarian PVY^{NTN} isolate as well as with both the Nicola clones (Fig. 3). It should be stressed that both the Wilga clones differed by 9 and 6 point mutations from the Hungarian isolate of PVY^{NTN} and the Nicola P1/1 clone, respectively (Fig. 3, A). Moreover, significant differences between PVY^{NTN} and the Wilga isolates were observed at amino acid level (Fig. 3, B), especially at aa 74, where hydrophobic, positively charged Arg in PVY^{NTN} was substituted by polar, negatively charged Gln in the Wilga pathotype, which could change the P1 properties. It is not clear whether or not the sequence variants of the Wilga pathotype represent in fact a mixture of different isolates which appeared due to multiple infection, unless single lesion inocula are prepared and analyzed. On the other hand, it can be assumed that minor sequence variants, which are often seen in PVY isolates, represent minor fraction(s) of mutated molecules which appear during virus replication and could form virus quasispecies (Clarke *et al.*, 1994). RT-PCR-amplified quasispecies were, e. g., described by Mcneil *et al.* (1996) for the protein coding region and the 3'-non-coding region of the genome of wheat streak mosaic rymovirus. Comparison of restriction maps of cloned virus genomes revealed considerable variability within strains of beet curly top virus (Stenger and McMahon, 1997). A significant sequence variability was demonstrated for many of other plant viruses (e.g. Kurath *et al.*, 1993; Moya *et al.*, 1993; Chalhoub *et al.*, 1994; Bousalem *et al.*, 1994; Chenault *et al.*, 1996; Magome *et al.*, 1997).

It is obvious that methods like TGGE or DNA heteroduplex analysis used here represent useful and powerful tools, which could help together with RFLP analysis and other methods to solve the problems of virus variability and analysis of mixed virus infections.

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