Acta Phytopathologica et Entomologica Hungarica 39 (4), pp. 315–324 (2004)

Sequence Variants of Potato Virus Y in Field Grown Tobacco with Different Resistance Background Including Transgenic Ones

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Experimental fields of a tobacco breeding station in Hungary at Debrecen were surveyed for potato virus Y (PVY) populations for over a decade. In this field, hundreds of crosses were grown regularly and most of them were lines and hybrids genetically resistant against potato virus Y. From 1993, transgenic tobacco lines bearing the coat protein gene of the new tuber necrotic strain of potato virus Y (NTN) were planted in this experimental field and tested yearly for their virus resistance and the durability of their resistance. During this period, no peculiar strain of PVY was observed, judging from symptoms or using biological tests. In the last five year period the biological observations of PVY isolates were combined with partial molecular analysis. The coat protein gene of PVY from all different isolates collected yearly was cloned, and the most variable 5' ends of the genes were sequenced and compared. All isolates belonged to the new tuber necrotic strain except one, showing the relative stability of the potato virus Y population in an area where genetically different tobacco lines, including transgenic ones, are grown and exposed to natural infection.

Keywords: potato virus Y, transgenic resistance, field experiments population stability.

Since the first transgenic virus-resistant tobacco plant was engineered by integrating the coat protein gene of *Tobacco mosaic virus* (Powell-Abel et al., 1986) dozens of other plants were successfully transformed with other plant virus coat protein genes, achieving the same agronomically important trait (Lomonosoff, 1995). This coat-protein-mediated approach has been extended by using other viral genes obtaining similar resistance, which was in accordance with the concept of pathogen-mediated protection (Sanford and Johnston, 1985). Although the mechanisms of the resistance could be different, based either on the accumulation of the coat protein or on a gene-silencing phenomenon, it is obvious that genetic engineering has revolutionized plant virus resistance breeding. Such genetically engineered virus-resistant papaya plants are already a great economic success (Gonsalves, 2003). The increase in cultivation of transgenic virus-resistant crop all over the world (James, 2002), along with the biosafety concerns regarding these specific engineered plants, highlighted the importance of experimental study of their potential ecological impacts (Tepfer and Balázs, 1997; Tepfer, 2002). One of the most important is the stability of the virus population in open environmental conditions when and where plants bearing

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viral sequences are grown. Potato virus Y (PVY) is the type member of the Potyviridae family, the largest family of plant viruses, infecting hundreds of agronomically important crops (Shukla et al., 1994). PVY is a monopartite virus with a positive-sense single-stranded RNA genome, which is translated into a large polyprotein and later processed into nine functional proteins. PVY is one of the most serious viral pathogens of *Solanaceous* species like potato, tomato and tobacco, and most of the strains were originally isolated from those crops. The separation of the two main groups of isolates is based on the symptoms induced on tobacco. The severe necrotic strains became abundant from the 80’s in Europe after tuber necrosis symptoms were described first (Horváth, 1967, Beczner et al., 1984). Since that time, the necrotic isolates are dominant in the Hungarian flora. Virus recombination is a driving force of their existence (Simon and Bujarski, 1994). In transgenic plants, where viral sequences are present in the plant cells, RNA recombination has new environmental roles. There are several reports on the detection of recombinant viruses in non-transgenic and transgenic plants in experimental conditions both with selection pressure and without (Aaziz and Tepfer, 1999 a,b). Recombinant viruses, made *in vitro* help us understand viral gene functions and disease symptom development (Schoelz and Wintermantel, 1993), and sequence studies reveal that some peculiar isolates originates from natural recombination of distinct isolates (Cervera et al., 1993; Tepfer, 2002).

These important findings and the field experiments of the transgenic PVY resistant tobacco hybrids and lines inspired us to survey the PVY population in an area where transgenic tobaccos are grown regularly, and to monitor the potential emergence of a new virus strain or pathotype.

Materials and Methods

Plant material

Tobacco plants were regularly monitored for the presence of PVY. Potato virus Y coat protein gene expressing tobacco transformant lines were grown for testing their virus resistance in field conditions. Transformation experiments were described elsewhere (Kollár et al., 1993) including their field performance (Józsa et al., 2002). Samples were collected from different naturally infected tobacco cultivars, breeding lines and from transgenic ones between 1999 and 2003. Virus isolates were collected in the second week of August when virus induced symptoms were clearly manifested *(Table 1).* Virus isolates were transfected to *Nicotiana tabacum* L. cv. Xanthi in the greenhouse, from which the nucleic acids were isolated. The field experiment of transgenic tobacco was permitted by the competent authority licence number 5470/03/2000.

Partial molecular characterisation of the isolates
RNA purification, cDNA synthesis, and polymerase chain reaction, (PCR)

Total RNA was extracted from 25 milligrams (two small leaf discs) of tobacco leaf using the method of White and Kaper (1989). Ethanol precipitated total RNA was redissolved in 60 µl of water, 2 µl was used for reverse transcription in 10 µl RT reaction mix

Table 1

Genetic background of tobacco varieties and breeding lines in relation of PVY resistance. After the code the year of the isolation marked

Host name/code	PVY resistance/susceptibility
B21 Burley 21	Susceptible
Coker 254	Susceptible
Hevesi 1	Resistant (va gene)
Hevesi 9	Resistant (va gene)
Hevesi 12	Resistant (va gene)
Hevesi 17	Resistant (va gene)
K326	Susceptible
McNair 944	Susceptible
NC95	Susceptible
NCTG 52	Resistant (VR gene)
Pallagi 1	Resistant (va gene)
PBD 6	Resistant (va gene)
TN 86	Resistant (va gene)
VAM	Resistant (va gene)
VD 17	Resistant (transgenic)
Virginia SCR	Resistant (va gene)

using universal polyT primer (5'-CGGGGATCCTCGAGAAGC(T)17-3') (Deborré et al., 1995). PVY-specific products (containing the coat protein gene and the 3' UTR) were amplified by PCR using a Gene Amp PCR System 9700 thermal cycler (Applied Biosystem) using degenerate primer PVYCP5' (5'-GGGAATTCCGCGGAAATGACACAAT (CT)TGATGCAGGAG-3') and polyT primer in 50 µl volume. The reaction mixture contained 3 µl of cDNA, 20 pmol of primers, 200 µmol of each dNTP, PCR reaction buffer and 2.5 U Taq polymerase (Fermentas). Denaturation at 94 °C for 3 minutes was followed by 40 cycles of 15 seconds at 94 °C denaturation, 30 seconds at 45 °C annealing, 3 minutes at 72 °C extension, with a final extension step at 72 °C for 10 minutes.

Cloning and sequence analysis.

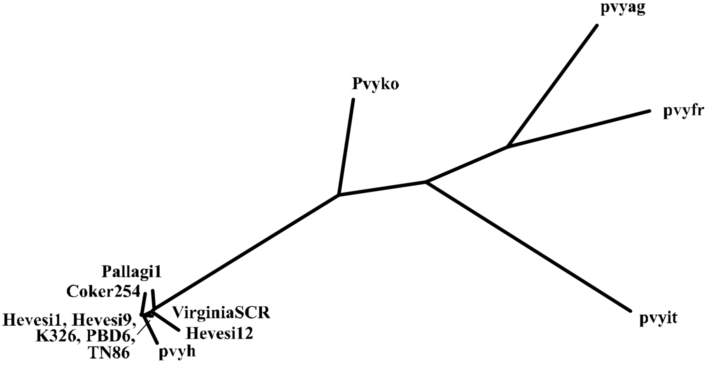
The amplified DNA fragments were purified using phenol and chloroform. Ethanol precipitated PCR products were redissolved in water and were digested with SacII and BamHI endonucleases (the restriction sites were introduced in the primer sequence). Inserts of approximately 1200 bp were separated on 1% agarose gel using DNA purification kit (Bio-Rad), and were cloned into a pBluescript SK+ plasmid (Stratagene). The recombinant plasmids were transformed into *Escherichia coli* DH5α (Sambrook et al., 1989).

From the cloned virus fragments 371 nt at the 5' end hypervariable region of the coat protein gene were sequenced (26-396 nt on the PVY CP gene) with ABI Prism automated DNA sequencer. Sequence analysis was performed using University of Wisconsin Genetics Computer Group (GCG) sequence analysis software package version 9.1. Multiple sequence alignments were generated using PILEUP of GCG package. CLUSTALW was used to create an input file for PHYLIP. The tree was displayed using DRAWTREE program.

Results and Discussion

During the last five years 59 PVY isolates were collected at Debrecen (Hungary) in the experimental field of the tobacco breeding institute. After transfecting these to *Nicotiana tabacum* L. cv. Xanthi the viral RNAs were amplified, and their 3' end including the coat protein gene was cloned. The most variable fragment of the coat protein gene was selected for cloning and for sequencing and analysis of sequence homology. This fragment was specifically selected for that simple reason that the transgenic plants grown on the given field bear the coat protein message in their genome, so the sequence could have a direct role in the change of the virus population via complementation or recombination. The sequences were compared and their phylogenetic trees are shown in *Figs 1, 2, 3.* As all the sequences used for comparison belong to the necrotic group or new tuber necrotic group of the PVY strains their high sequence homology is good evidence that all isolates are very close to the H isolates, which are dominant in the Hungarian flora (Thole et al., 1993), except one in 2000 has higher similarity to the French isolate (strain N) than to the Hungarian strain. No symptom difference or peculiarity was observed except that symptom severity and development varied among the isolates. Virus accumulation was not analyzed, but differences in virus titer could contribute to any differences in symptomathology.

A



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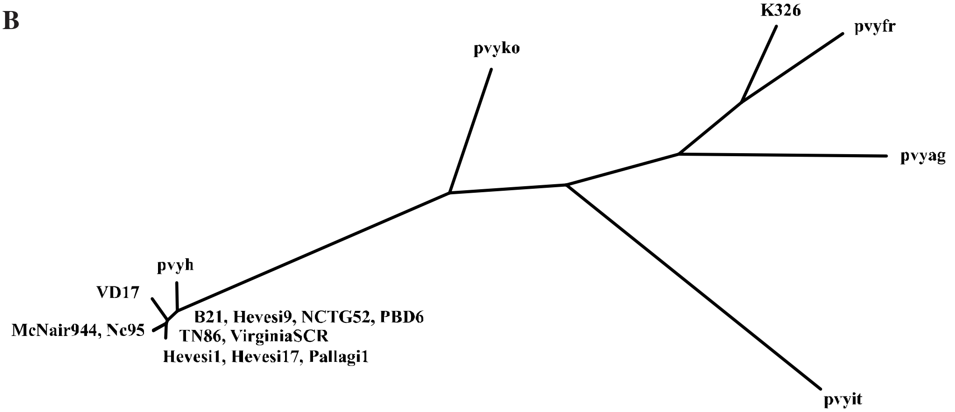

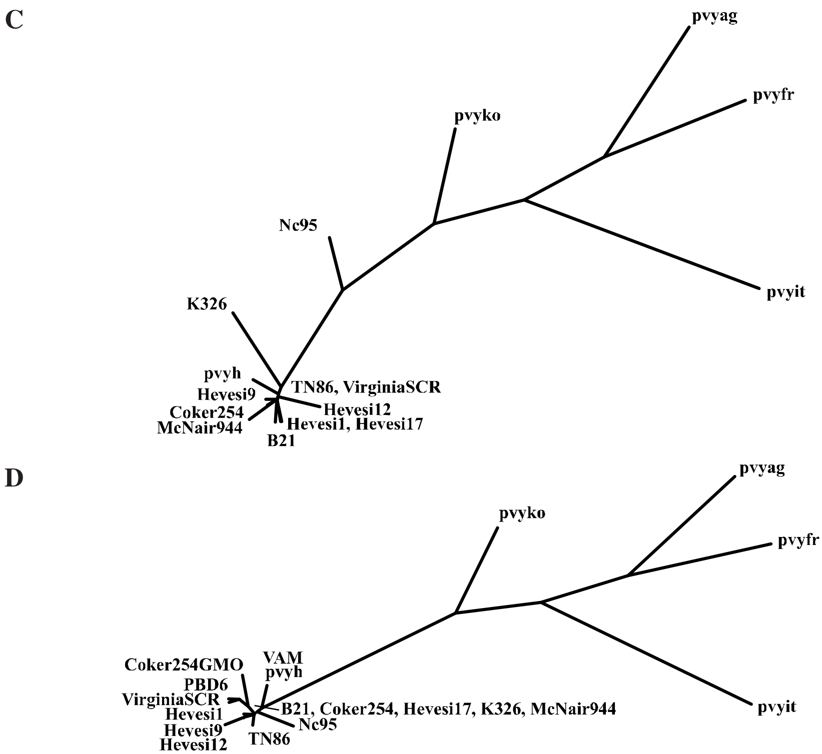

Fig. 1. Phylogenic trees of the PVY isolates collected yearly between 1999-2002. (A/1999, B/2000, C2001, and D, 2002) The comparison was made on the sequenced first 371 nt hipervariable region of the 5' end of the coat protein gene and the sequence analysis was performed using University of Wisconsin Genetics Computer Group sequence analysis software package version 9.1. Multiple sequence alignments were generated using PILEUP of GCG package. CLUSTALV was used to create an input file for PHYLIP. Reference sequences were downloaded from EMBL databank. Pvyit correspond to an Italian necrotic PVY isolate, pvyfr to the necrotic French PVY isolate, while pvyag an Argentinian and pvyko a Korean necrotic isolate of the virus (bold). Sequences of this work deposited in the EMBL data library under the following accession numbers:

Code for the local isolates correspond to the host tobacco lines and cultivars having different resistance background for PVY resistance. See *Table 1.*

Accession#: AJ581301 Description: Potato virus Y partial CP gene for coat protein, isolate B21-2000,
Accession#: AJ581302 Description: Potato virus Y partial CP gene for coat protein, isolate B21-2001,
Accession#: AJ581303 Description: Potato virus Y partial CP gene for coat protein, isolate B21-2002,
Accession#: AJ581304 Description: Potato virus Y partial CP gene for coat protein, isolate Coker254 1999,
Accession#: AJ581305 Description: Potato virus Y partial CP gene for coat protein, isolate Coker254 2001,
Accession#: AJ581306 Description: Potato virus Y partial CP gene for coat protein, isolate Coker254 2002,
Accession#: AJ581307 Description: Potato virus Y partial CP gene for coat protein, isolate Coker254GMO-002,
Accession#: AJ581308 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi1 1999,
Accession#: AJ581309 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi1 2000,
Accession#: AJ581310 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi1 2001,

Accession#: AJ581311 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi1 2002, Accession#: AJ581312 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi9 1999, Accession#: AJ581313 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi9 2000, Accession#: AJ581314 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi9 2001, Accession#: AJ581315 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi9 2002, Accession#: AJ581316 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi12 1999, Accession#: AJ581317 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi12 2001, Accession#: AJ581318 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi12 2002, Accession#: AJ581319 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi17 2000, Accession#: AJ581320 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi17 2001, Accession #: AJ581321 Description: Potato virus Y partial CP gene for coat protein, isolate Hevesi17 2002, Accession#: AJ581322 Description: Potato virus Y partial CP gene for coat protein, isolate K326-1999, Accession#: AJ581323 Description: Potato virus Y partial CP gene for coat protein, isolate K326-2000, Accession#: AJ581324 Description: Potato virus Y partial CP gene for coat protein, isolate K326-2001, Accession#: AJ581325 Description: Potato virus Y partial CP gene for coat protein, isolate K326-2002, Accession#: AJ581326 Description: Potato virus Y partial CP gene for coat protein, isolate McNair944 2000, Accession#: AJ581327 Description: Potato virus Y partial CP gene for coat protein, isolate McNair944 2001, Accession#: AJ581328 Description: Potato virus Y partial CP gene for coat protein, isolate McNair944 2002, Accession#: AJ581329 Description: Potato virus Y partial CP gene for coat protein, isolate Nc95-2000, Accession#: AJ581330 Description: Potato virus Y partial CP gene for coat protein, isolate Nc95-2001, Accession#: AJ581331 Description: Potato virus Y partial CP gene for coat protein, isolate Nc95-2002, Accession#: AJ581332 Description: Potato virus Y partial CP gene for coat protein, isolate NCTG52 2000, Accession#: AJ581333 Description: Potato virus Y partial CP gene for coat protein, isolate Pallagi1 1999, Accession#: AJ581334 Description: Potato virus Y partial CP gene for coat protein, isolate Pallagi1 2000, Accession#: AJ581335 Description: Potato virus Y partial CP gene for coat protein, isolate PBD6-1999, Accession#: AJ581336 Description: Potato virus Y partial CP gene for coat protein, isolate PBD6-2000, Accession#: AJ581337 Description: Potato virus Y partial CP gene for coat protein, isolate PBD6-2002, Accession#: AJ581338 Description: Potato virus Y partial CP gene for coat protein, isolate TN86-1999, Accession#: AJ581339 Description: Potato virus Y partial CP gene for coat protein, isolate TN86-2000 Accession#: AJ581340 Description: Potato virus Y partial CP gene for coat protein, isolate TN86-2001, Accession#: AJ581341 Description: Potato virus Y partial CP gene for coat protein, isolate TN86-2002, Accession#: AJ581342 Description: Potato virus Y partial CP gene for coat protein, isolate VAM-2002, Accession#: AJ581343 Description: Potato virus Y partial CP gene for coat protein, isolate VD17-2000 , Accession#: AJ581344 Description: Potato virus Y partial CP gene for coat protein, isolate, VirginiaSCR-1999, Accession#: AJ581345 Description: Potato virus Y partial CP gene for coat protein, isolate VirginiaSCR-2000, Accession#: AJ581346 Description: Potato virus Y partial CP gene for coat protein, isolate VirginiaSCR-2001, Accession#: AJ581347 Description: Potato virus Y partial CP gene for coat protein, isolate VirginiaSCR-2002, Accession#: AJ581301 Description: Potato virus Y partial CP gene for coat protein, isolate B21-2000, Accession#: AJ581302 Description: Potato virus Y partial CP gene for coat protein, isolate B21-2001, Accession#: AJ581303 Description: Potato virus Y partial CP gene for coat protein, isolate B21-2002, Accession#: AJ581304 Description: Potato virus Y partial CP gene for coat protein, isolate Coker254 1999, Accession#: AJ581305 Description: Potato virus Y partial CP gene for coat protein, isolate Coker254 2001, Accession#: AJ581306

As several environmental factors can exert evolutionary constraints on PVY, of which are of the most important is the host plant. Our finding on the sequence stability in the population of PVY over five years, and a similar dominance of the necrotic PVY strains on potato field in Germany (E. Maiss, personal communication 2003), suggest that selection and evolution of PVY isolates depends on the cultivated crop varieties. As today

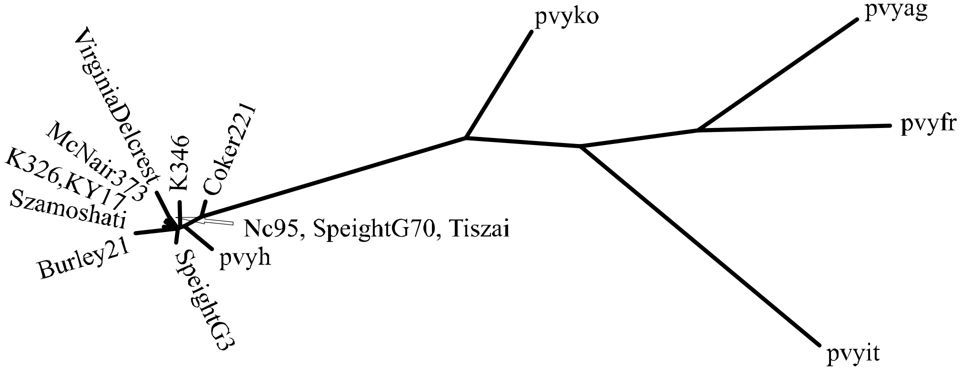

Fig. 2. Phylogenic tree of the PVY isolates from 2003. (Nc95, SpeightG70, Tiszai, SpeightG3, Burley21, Szamosháti, K326, KY17, McNair373, VirginiaDelcrest, K346, Coker221)

most of the grown tobacco cultivars are resistant to PVY strains, mainly the strong resistance-breaking necrotic strains could be detected in fields. Mild strains, like members of PVY O, group are quite rarely found. Moury et al. (2002) conclude, from their sequence compilation data on potyvirus host adaptation, that, except in the case of PVY, the coat protein is not involved as a host range determinant. Since coat protein of PVY has several functions in the virus life cycle, including aphid transmissibility which is a key factor of virus spread and disease epidemiology, the presence and expression of the coat protein message in the plant genome could have a direct role in those functions, and could contribute to the evolution of new sequence variants, with new pathological properties. The sequence comparison of seven new tuber necrotic isolates showed the same high sequence homology in the studied region of the 5' end of the CP (Cerovska et al., 2001), suggesting that NTN isolates are highly conserved in that region and diverged from other pathotypes. This emphasizes the importance of this region in the pathogenicity of the virus isolates.

Conclusions

Surveillance of the PVY population over a decade in the experimental field of a tobacco breeding station in Hungary showed a relative stability in nature in the virus population. During a five years period, molecular analysis of the sequence variants showed the dominant presence of the necrotic strains in a field where different genetically resistant lines and hybrids were grown, including transgenic PVY coat protein bearing plants. Although the time scale of the observations is rather short, the results suggest that the presence of transgenic plants in the field were not affected the genetic variability of the population among the sequence analysed. We have to underline also, that mutation occurred in other part of the genomes, which could lead the formation of a new pathotype but the pathological observation of this field have no indication that mutation happened by resulting a different phenotype.

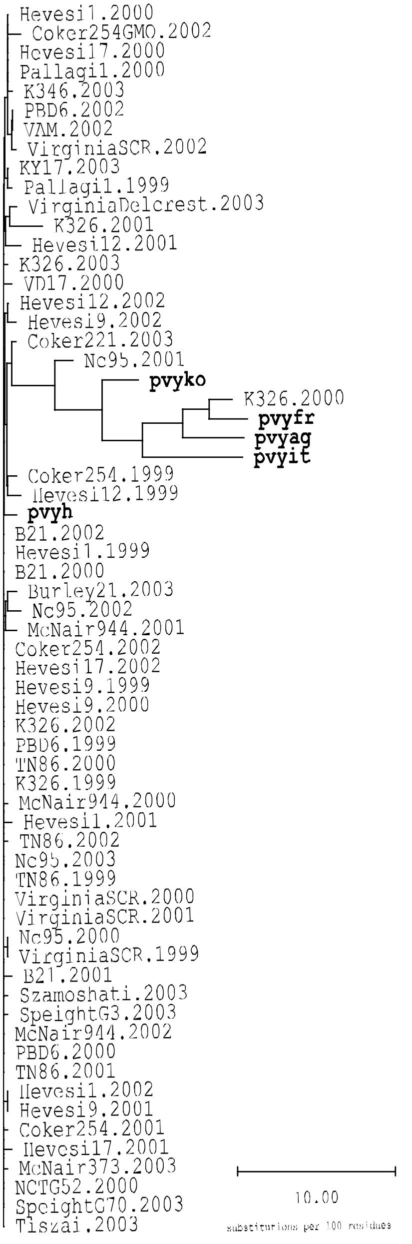

Fig. 2. Phylogenic trees of the PVY isolates collected yearly between 1999–2003. The comparison was made on the sequenced first 371 nt hipervariable region of the 5' end of the coat protein gene and the sequence analysis was performed using University of Wisconsin Genetics Computer Group sequence analysis software package version 9.1. Multiple sequence alignments were generated using PILEUP of GCG package. DISTANCES program was used to create an input file for GROWTREE

Acknowledgements

This work was supported in part by EC grant QLK3 –CT – 2000-00361 (VRTP IMPACT). Ruslan Kryldakov (University of Kazakhstan, Almaty) was a recipient of UNESCO BETCEN fellowship. László Palkovics was supported by János Bolyai fellowship of the Hungarian Academy of Sciences. Scientific schools also supported the work (OTKA TS 44778). The valuable help of Dr. Gyula Nagy (Agrotab LTD, Debrecen) in the isolate collection is highly appreciated. Ervin Balázs was awarded with Blaise Pascal International Research Chair founded by the Ecole Normale Supèrieure, the French Governement and the Region Ile de France.

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