

## Restriction analysis of genetic variability of Polish isolates of *Tomato black ring virus*<sup>✉\*</sup>

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Several different isolates of *Tomato black ring virus* (TBRV) have been collected in Poland from cucumber, tomato, potato and black locust plants. Biological tests showed some differences in the range of infected plants and the type of symptoms, which was the basis for selection of seven the most biologically different TBRV isolates. According to the sequence of TBRV-MJ, several primer pairs were designed and almost the entire sequence of both genomic RNAs was amplified. The RT-PCR products derived from all tested TBRV isolates were digested by restriction enzymes. On the basis of the restriction patterns, the variable and the conserved regions of the TBRV genome were defined and the relationships between the Polish TBRV isolates established.

*Tomato black ring virus* (TBRV) belongs to the *Nepovirus* genus of positive, single stranded RNA plant viruses. Its genome is divided into two RNAs: RNA1 containing the genes important for virus replication and polyprotein processing and RNA2 containing genes responsible for the synthesis of viral capsid protein and the movement of the virus in plants (Demangeat *et al.*, 1990; 1991; Grief

*et al.*, 1988; Mayer *et al.*, 1986; Le Gall *et al.*, 1995a). Earlier, some Polish TBRV isolates were found in potato (Chrzanowska & Śniegowski, 1965), tomato (Twardowicz-Jakusz, 1969), celery (Twardowicz-Jakusz, 1976), carrot (Twardowicz-Jakusz *et al.*, 1977a), horseradish (Twardowicz-Jakusz *et al.*, 1977b), privet (Błaszczak & Pospieszny 1987), forsythia (Kamińska & Sobilo, 1983),

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\* Accession numbers for TBRV-MJ full-length sequence of RNA1: NC 004439 and RNA2: NC 004440.

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**Abbreviations:** GFLV, *Grapevine fanleaf virus*; nt, nucleotides; TBRV, *Tomato black ring virus*.

gladiolus and flox (Kamińska & Woś, 1978). Those isolates, except the one from potato, are no longer available. Recently, we have collected some new TBRV isolates in Poland. Most of them were isolated from old black locust plants (Pospieszny & Borodynko, 1999; Borodynko *et al.*, 2001), one isolate from tomato (Pospieszny & Borodynko, 1999) and one from cucumber (Pospieszny *et al.*, 2003). In contrast to their biological variability, all the Polish TBRV isolates tested appeared serologically similar to each other (Borodynko *et al.*, 2001).

RNA viruses have a high capability of rapid genetic changes due to the frequent point mutations and genome rearrangement that are held to be the main mechanism in the evolution of RNA viruses (Drake 1993; Dolja & Carrington, 1992; Domingo & Holland, 1997; Roossinck, 1997). The majority of the point mutations in the viral genomes are neutral for the encoded amino-acid sequence but probably they might influence the RNA structure and adaptation to the host translation machinery (Huynen *et al.*, 1996; Leisner & Neher, 2002; Roossinck, 2002). RNA recombination might be responsible for the viral sequence variability, repair of some defective RNA molecules as well as production of shortened form of viral RNA, called defective interfering RNAs (Bruyere *et al.*, 2000; Kim & Kao 2001; Nagy & Bujarski, 1998). These truncated RNA molecules might interfere with virus replication and change the symptoms severity in infected plants (Graves *et al.*, 1996; Hernandez *et al.*, 1996; Cheng *et al.*, 2002; Szittyta *et al.*, 2002).

The variability of RNA viruses is also connected with specified parts of their genomes. Generally, the most conservative is the region encoding polymerase (Dolja & Carrington, 1992; Zaccomer *et al.*, 1995; Koonin 1991). The coat protein gene is usually variable and virus species specific, and due to its responsibility for the specification of the viral serotype and species it is frequently used in phylogenetic analyses (Dolja & Carrington,

1992; Zaccomer *et al.*, 1995; Petrzik & Lenz, 2002; Canizares *et al.*, 2001). Many other viral proteins, especially those of unknown function, are only rarely used in viral phylogeny due to their often one-species-specific nature and a lack of counterparts in other viral species (Zaccomer *et al.*, 1995).

The knowledge of virus population diversity and the distribution of the variable and conserved regions within the viral genomes may be useful in sequence-specific detection of viruses, the prediction of occurrence of resistance-breaking viral phenotypes as well as in developing new methods of plant protection.

In this paper we report the genetic variability of Polish TBRV isolates on the basis of restriction analysis of almost full-length cDNA derived from both viral RNAs.

## MATERIALS AND METHODS

**TBRV isolates.** The TBRV isolates studied originated from the following plant species: cucumber (TBRV-Cuc), tomato (TBRV-Tom), black locust (TBRV-L10, TBRV-MJ, TBRV-N1, TBRV-Pn) and potato (TBRV-Pot). TBRV-Pot was kindly supplied by Prof. M. Chrzanowska (IHAR, Radzików, Poland), the other isolates were from our collection. The biological properties of the TBRV isolates were studied by mechanical inoculation of various plant species. The host range and symptoms allowed selection of the isolates displaying the most distinct properties. All the isolates were maintained and propagated in *Chenopodium quinoa* in the greenhouse.

**Purification of viral RNA.** The TBRV isolates were propagated in *C. quinoa* and purified as described before (Pospieszny & Borodynko, 1999). The purified viral particles were digested with proteinase K and RNA was isolated according to the phenol/chloroform protocol (Sambrook *et al.*, 1989), precipitated with 96% ethanol and pellet was dissolved in RNase-free water, then 2  $\mu$ l of RNA solution were mixed with 10  $\mu$ l of Formazol

(MRC) and separated on 1% agarose non-denaturing gel.

**RT-PCR amplification of RNA1 and RNA2.** The purified viral RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) according to manufacturer's instructions. The primer used for the first strand synthesis was oligo d(T)<sub>22</sub> and the full-length cDNAs obtained were amplified using four pairs of primers designed according to the TBRV-MJ sequence (Accession number: NC 004439 and NC 004440 for RNA1 and RNA2, respectively). The first primer pair (P-128 and 1MP2) amplified the 5' part of RNA1 about 2900 nt in length, the second one (1MP3 and 3ter) about 3600 nt from the 3' part of RNA1, the third (P-128 and 2MP2) the 5' part of RNA2 of about 3200 nt and the last pair (2MP5 and 3ter) amplified the 3' part of RNA2 about 2300 nt in length (Table 1). The obtained PCR products covered the entire coding sequence of RNA2 and almost entire sequence of RNA1, excluding about 800 nt from the region coding for NTP-binding protein. The amplification of cDNA obtained from seven Polish TBRV isolates was performed using ExpandLong PCR System (Roche) according to the manufacturer's instruction.

**Restriction digestion of PCR derived products.** The restriction enzymes were chosen on the basis of TBRV-MJ sequence using the MapDraw program from the DNA Star package (kindly made available by Institute of Biochemistry and Biophysics, Polish Acad-

emy of Sciences, Warszawa, Poland). Each RT-PCR product was digested by two different enzymes in two distinct restriction experiments and the digestion products were resolved on 2.5% NuSieve 3:1 agarose gel (BMA). The patterns obtained were analyzed in two aspects: firstly, a comparative analysis of conserved and variable regions was performed according to the bands of known size derived from TBRV-MJ and secondly, on the basis of the restriction fragments, 124 characters for phylogenetic analysis of the Polish TBRV isolates were obtained. The clustering and genetic distances assessment was performed using the program FreeTree (Pavlicek *et al.*, 1999). The phylogeny tree was bootstrapped 1000 times and constructed using the TreeView program (Page, 1996). The genomic mapping of the restriction bands obtained was established by comparison with TBRV-MJ patterns.

## RESULTS

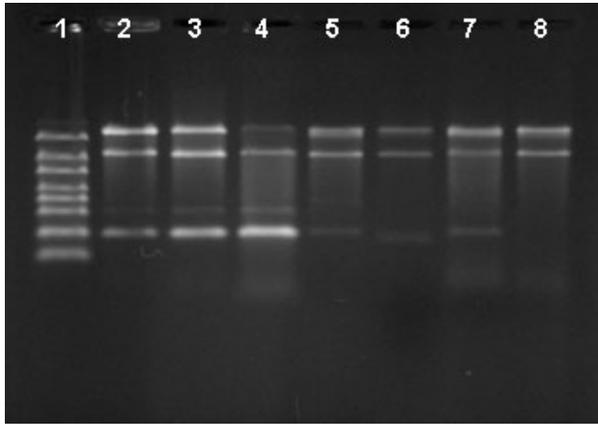
### Analysis of viral RNA

Analysis of purified viral RNA showed two RNA bands of a size typical for TBRV. For the majority of the isolates some additional bands of small non-genomic RNA appeared (Fig. 1). The origin of these small RNAs is unclear. The RNAs expected for TBRV satellite are about 1350 nucleotides in length (Hemmer *et al.*, 1993; Fritsch *et al.*, 1984; 1993) and the

**Table 1. The sequences of used primers**

Primer	Sequence
P128 (+)	caa atc ctg taa cca act ag
3ter (-)	(t) <sub>18</sub> ttg ctt ttt gca gaa aac att tta tca tat aca aa
1MP2 (-)	tgg att ttc cgg gtc ata ga
1MP3 (+)	ttc tgg atg ggg att ctg g
2MP2 (-)	tgg gat atg tca atg ggt tc
2MP5 (+)	act tca ggg ctt tcc gct

observed bands are mostly smaller. We also observed that those small, non-genomic RNAs could emerge during prolonged propagation of the isolates originally devoid of them (not shown).



**Figure 1.** Electrophoretic patterns of RNAs of Polish TBRV isolates.

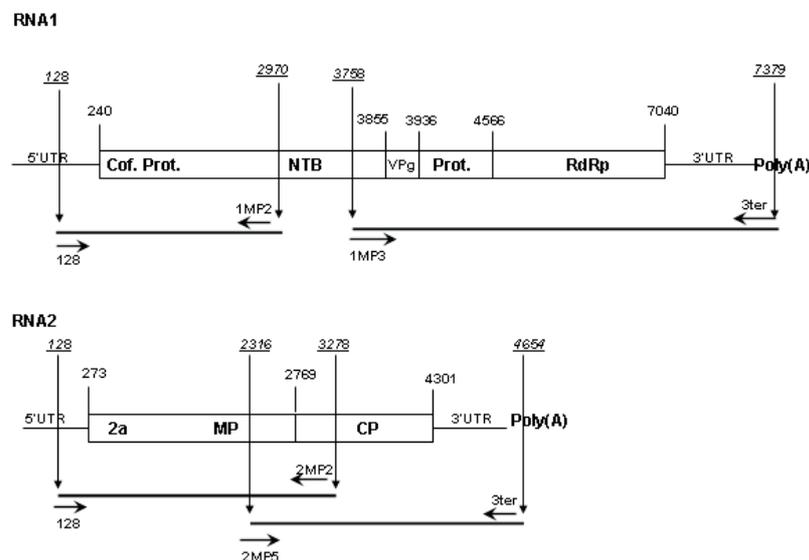
Lane 1, RNA ladder (6000, 4000, 3000, 2000, 1500, 1000, 500, 200 nt); lane 2, TBRV-L10; lane 3, TBRV-MJ; lane 4, TBRV-N1; lane 5, TBRV-Cuc; lane 6, TBRV-Tom; lane 7, TBRV-Pn; lane 8, TBRV-Pot.

### RT-PCR amplification of RNA1 and RNA2

In RT-PCR amplification four products were obtained for each tested TBRV isolate. Two of them correspond to the RNA1 sequence and two others to RNA2. All the products obtained were of comparable size. The putative organization of the RT-PCR products is shown in Fig. 2. The displayed numbers of nucleotides corresponding to the cleavage sites for both TBRV-MJ polyproteins have been discussed elsewhere (Jończyk *et al.*, 2004).

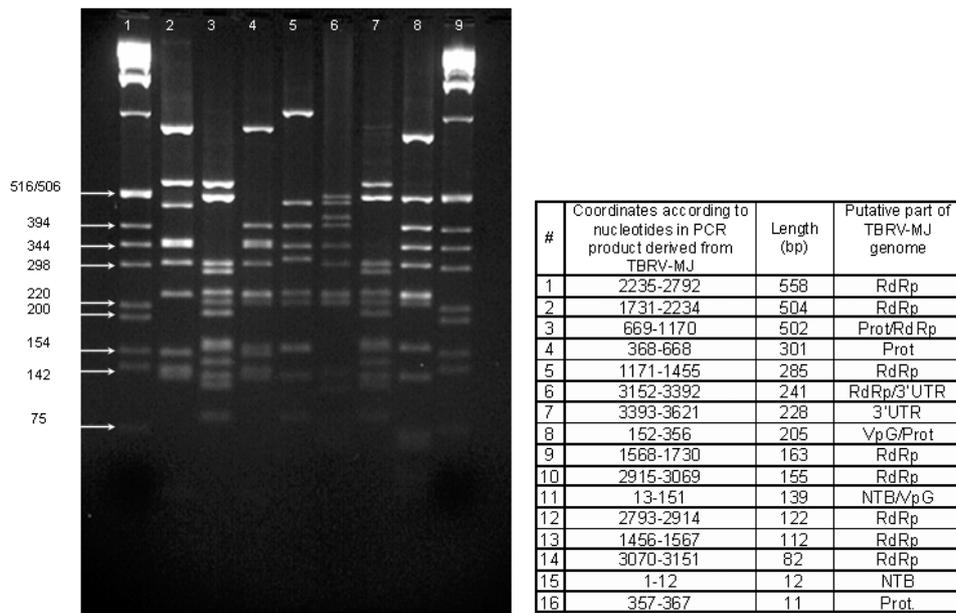
### Restriction digestion of PCR derived products

Restriction analyses of the RT-PCR products showed differences among the TBRV isolates. A higher diversity in the restriction patterns was observed for RNA1 and the restriction patterns were more diverse for the RT-PCR products corresponding to the genes in the 5' end proximity of both genomic RNAs. Figures 3a and 3b show examples of the restriction patterns of the isolates in com-



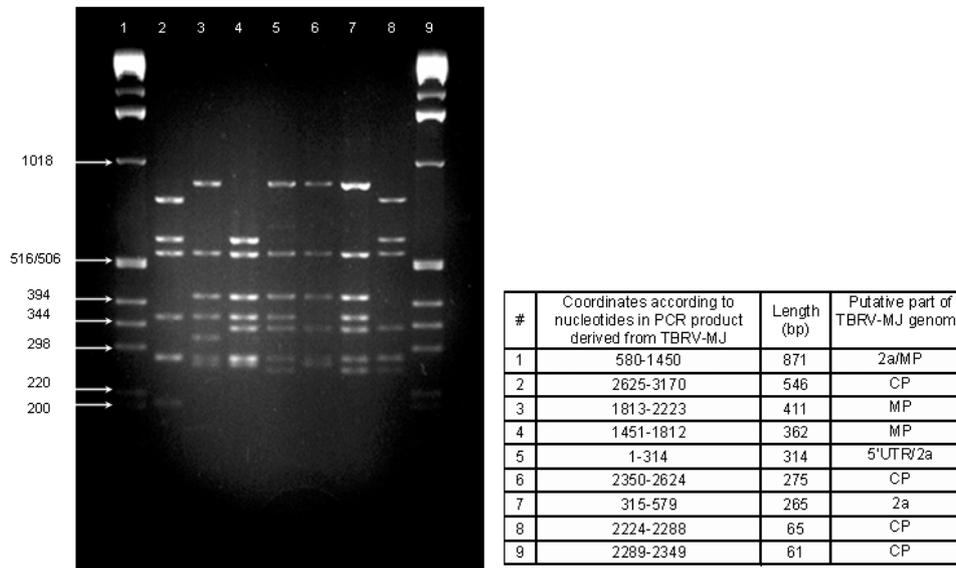
**Figure 2.** TBRV genome organization and location of primers and RT-PCR products.

RdRp, RNA-dependent RNA polymerase; Cof.Prot, protease cofactor; NTB, NTP-binding protein; Prot, protease; 2a, 2a protein; MP, movement protein; CP, coat protein. The numbers presented in regular numerals indicated the nucleotides corresponding to putative cleavage sites for TBRV polyproteins and the numbers in italics and underlined correspond to the sites of primer annealing.



**Figure 3a.** RT-PCR products of the 3' part of RNA1 digested by *Hinf*.

Lane 1 and 9, 1 Kb DNA ladder (GibcoBRL); lane 2, TBRV-L10; lane 3, TBRV-MJ; lane 4, TBRV-N1; lane 5, TBRV-Cuc; lane 6, TBRV-Tom; lane 7, TBRV-Pn; lane 8, TBRV-Pot.



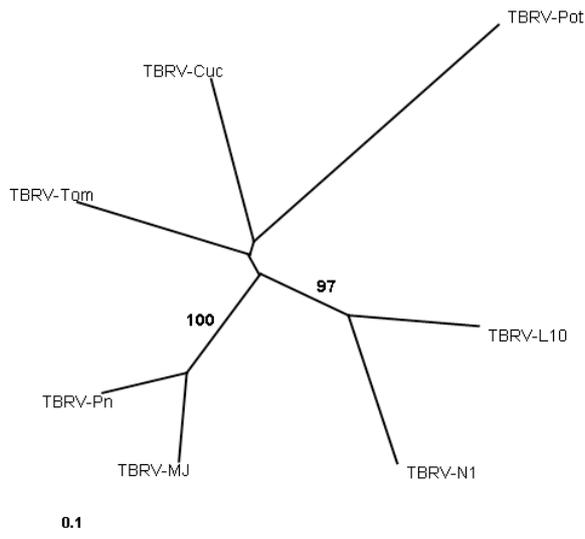
**Figure 3b.** RT-PCR products of the 5' part of RNA2 digested by *Hinf*.

For details see legends to Fig. 3a.

parison with those of TBRV-MJ for which the full-length sequence of both RNAs was already known.

The obtained restriction patterns allowed creation of a dendrogram of the relationship for the Polish TBRV isolates (Fig. 4). The re-

sulting tree was not supported by high bootstrap values for the isolates from crops. Four TBRV isolates from black locust were clustered into two pairs: TBRV-Pn with TBRV-MJ and TBRV-N1 with TBRV-L10. The genetic distance (Nei and Li) within a pair was much lower (e.g. 0.20879 between TBRV-MJ and TBRV-Pn) than between the pairs (e.g. 0.5713 between TBRV-N1 and TBRV-MJ). The iso-



**Figure 4.** Dendrogram of the relationships between Polish TBRV isolates.

lates from crop plants were more similar to each other than to the isolates from black locust. The most different among the isolates originating from crops was TBRV-Pot.

## DISCUSSION

The Polish TBRV population is an interesting example of RNA virus diversity in a few plant species. The presented restriction analysis gave an overall view into the genetic variability of the isolates and allowed prediction of distribution of mutations within the TBRV genome. The restriction tests performed create a basis for phylogenetic analyses of the Polish TBRV isolates as well as permit a prediction of their likely evolution in Poland.

Contrary to our expectation, the restriction analyses showed diverse patterns for RNA1 and quite homogenous ones for RNA2. RNA1 encodes all the proteins thought to be highly conserved (e.g., RNA-dependent RNA polymerase or protease) and RNA2 encodes a capsid and a movement protein. While the movement protein is often considered conserved (Mushegian 1994; Melcher 2000), the coat protein usually varies within species (Steinkellner *et al.*, 1992; Petrzik & Lenz 2002; Canizares *et al.*, 2001). In fact, all Polish TBRV isolates were serologically similar, if not identical, in the tests performed such as ELISA or double-diffusion test (Borodynko *et al.*, 2001).

The sequences in the 5' end proximity of both RNAs, coding for a putative protease cofactor and 2a protein on RNA1 and RNA2, respectively, appeared to have more variable restriction patterns in comparison with the sequences in the 3' end proximity of the RNAs. The role of the former proteins in the TBRV life cycle is not clear. Protein 2a, similarly to 2a protein of *Grapevine fanleaf virus* (GFLV), might participate in replication of RNA2 (Gaire *et al.*, 1999) and the putative protease cofactor might modulate the activity of viral protease especially for polyprotein 2 maturation (Hemmer *et al.*, 1995). Unfortunately, the mechanisms of RNA2 replication and polyprotein 2 maturation are not completely understood for TBRV and it cannot be excluded that 2a protein and the putative protease cofactor have quite different functions in the TBRV life cycle.

Restriction analysis cannot provide information concerning amino-acid variability or their influence on the viral proteins' properties. However, it should be considered that even the silent mutations that do not influence the amino-acid sequence, can modulate RNA structure. Single nucleotide substitutions might also facilitate virus adaptation to the host translation machinery due to the correlation of the viral amino-acid codons with the ones used by the host plant (Roossinck

2002; Leisner & Neher, 2002). The tests performed allowed establishing the existence of a genetic diversity of the Polish TBRV isolates and indicated which part of their genomes probably gathered or/and maintained the diversity.

The phylogenetic relationships between the Polish TBRV isolates were established on the basis of restriction fragment length polymorphism analysis. The resulting dendrogram clustered the isolates from black locust into two groups and such clustering was supported by high bootstrap values (Fig. 4). The situation was different for the isolates from crops because the very low bootstrap values prevented us from identification of the most likely relationships.

Most of the Polish TBRV isolates have some additional RNA particles associated and encapsidated with the viral RNAs. Their atypical size and also abundant amount suggested their defective rather than satellite nature. This suggestion was supported by the observation that the small RNAs originated during serial passages of a non-genomic-RNA-free isolate in *C. quinoa* (not shown). The isolates after passages usually displayed decreased severity.

The best method for estimation of real variation among viral genomes is sequencing. However, this is usually done for a short part of the coding sequence and is typically limited to one or sometimes two most typical viral proteins. The restriction tests performed enabled a preliminary characterization of the Polish TBRV population according to almost entire genomic sequence and indicated in which part of the genome the variability was localized.

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