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Assessing the applicability of transcript conformation polymorphism for differentiation among *Prunus necrotic ringspot virus* isolates

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Summary. The applicability of single and double-stranded transcript conformation polymorphism (TCP) for differentiation among *Prunus necrotic ringspot virus* (PNRSV) isolates was evaluated and compared with other molecular differentiation procedures. Single-stranded TCP was found to be suitable to differentiate closely related virus isolates. However, due to its high variability, it was not suitable for grouping virus isolates. Double-stranded TCP, on the other hand, enabled the division of virus isolates into major groups. Restriction fragment length polymorphism (RFLP) of the PCR products confirmed the grouping of the virus isolates but this technique was limited in its ability to detect a wide range of nucleotide modifications. Nucleotide sequence analysis was essential for the detection of strain-specific sequences but did not clearly identify most other minor modifications that are necessary for virus classification. The combination of all methods is therefore sometimes required for complete analysis.

Key words: PNRS, strain differentiation, TCP, RFLP.

Introduction

Prunus necrotic ringspot virus (PNRSV) is a member of the genus *Ilarvirus* in the family *Bromoviridae* with a tripartite genome. PNRSV is disseminated worldwide in *Prunus* spp. (Fulton, 1983). There are numerous isolates or strains of the virus varying widely in their pathogenic, biophysical and serological properties (Aebig *et al.*, 1987). Distinguishing the various strains is, therefore, of prime importance.

Attempts to differentiate among PNRSV isolates have been made by several research groups. Mink *et al.* (1987) produced polyclonal antibodies that distinguished several serotypes of the virus. Crosslin & Mink (1992) demonstrated variations in the molecular weights of the coat protein of some PNRSV isolates.

Recent advances in molecular biology have provided new tools for the classification and identification of plant viruses. Strain-differentiation by means of restriction fragment length polymorphism (RFLP) analysis of PCR products has been carried out in several laboratories (Rosner *et al.*, 1998; Aparicio *et al.*, 1999). Determination of the nucleotide sequence of RNA3 for several PNRSV isolates (Guo *et al.*, 1995, Hammond and Crosslin, 1995; Scott *et al.*, 1998) enabled differentiation of several PNRSV isolates.

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In previous studies (Rosner *et al*., 1998, 1999), we differentiated PNRSV isolates by polymorphism of single and double-stranded RNA transcripts copied from amplified virus-specific PCR products. This method has the advantage of not requiring prior knowledge of the nucleotide sequence of the virus isolates. However, its unique contribution relative to other more established molecular methods has not been clearly defined.

In the present study we evaluated the relevance, importance and limitations of this method and compared it with other molecular procedures used to differentiate among PNRSV isolates.

Materials and methods

Virus isolates

Three isolates of PNRSV served as a model system in this study. Isolate Apr152 (Apr) was from apricot cv. Bergeron (Hungary), Ro from rose cv. Mercedes (Israel), and Pe30/4 (Pe) from peach (North Carolina, USA). These isolates were previously characterized by Rosner *et al*. (1998) and Spiegel *et al*. (1999). Three unknown isolates of PNRSV, M1, M2 and M3 originally introduced from Italy and grafted on peach trees in different regions of Turkey, were also included.

RNA extraction

Total RNA isolation from plant tissue (100 mg leaves) was carried out by means of the lithium method, avoiding phenol extraction, as detailed by Spiegel and Martin (1993).

PNRSV clones, primers and RT-PCR

The forward (I) and reverse (II) primers for PCR were as in Spiegel *et al*. (1999):
(I) 5'-GAGCTCTGGTCCCACTCAGG-3' (position 111-130);
(II) 5'-CTGCTTGAGATCTAGAGTGA-3' (position 701-720).

The T7 promoter (5'-AATTTAATACGACGACTCACTATA-3') was attached to primer I or II to yield PCR products with the promoter on its 5' or 3'-end respectively. The RNA of both orientations was thus obtained. A standard PCR protocol was applied. For details see Rosner *et al*. (1998, 1999).

Transcription

A 3-µl sample of the PCR product was incubated for one hour at 37°C together with 4 µl rNTP mixture (10 mM each), 4 µl transcription buffer ×5, 0.5 µl RNAsin (5 U μl^{-1} Promega, Madison, USA), 8.5 µl water (total volume 20 µl) and 1 µl T7 RNA polymerase (50 U μl^{-1}, New England, Biolabs, USA).

Preparation of double-strand transcript duplex

Two types of PCR products were made for each virus isolate, one with the T7 promoter on the 3'-end, the other with the promoter on the 5'-end. The PCR products were obtained by using, for amplification, either the forward primer I with the T7 promoter, or the T7 promoter attached to reverse primer II. Copying the two types of PCR products yielded RNA transcripts of two complementary orientations.

Annealing of RNA transcripts

Complementary RNA transcripts were mixed and incubated for 5 min at 50°C, followed by slow cooling to room temperature prior to gel electrophoresis.

Electrophoresis of RNA

One third by volume of loading buffer (50% glycerol, 1 mM EDTA and 0.4% bromophenol blue) was added to the RNA samples and fractionation was carried out by electrophoresis (applied voltage 100V) in a 10×8 cm, 6% polyacrylamide gel (PAG) in 40 mM Tris-acetate, pH 8.0; 1 mM EDTA (TAE) buffer at 25°C in a vertical minigel apparatus (BioRad Laboratories Inc., Hercules, CA, USA).

RFLP analysis of PCR products

Aliquots (6 µl) of the amplified PCR products were digested by the addition of 0.5 U of the appropriate restriction endonuclease to a recommended buffer in a total volume of 10 µl. The samples were incubated at 37°C for 2 h. The digests were fractionated in 6% PAG by electrophoresis.

Sequence analysis

PCR products of the virus isolates were cloned into pGEM-T Easy Plasmid vector (Promega) according to manufacturer's instructions. The standard forward (T7) and reverse (SP6) primers were used for sequencing the cloned virus fragment at the Molecular Biology Center Ltd. (MBC), Rehovot, Israel.

Results

The aim of this study was to assess the suitability of transcript confirmation polymorphism (TCP) together with two other molecular procedures: RFLP and nucleotide sequencing, for differentiation among PNRSV isolates. Three well-characterized isolates, Ro, Pe and Apr (Rosner *et al*., 1998; Spiegel *et al*., 1999) and three unidentified ones, M1, M2 and M3, were chosen for that purpose. These isolates were initially characterized by the more established methods of RFLP and nucleotide sequencing.

RFLP analysis of PCR products

A 600 bp fragment originating in the coat protein gene of PNRSV was amplified by PCR and analyzed by restriction cleavage (Fig. 1). Three of the virus isolates, M1, M2 and Pe, yielded similar cleavage patterns using *Rsa*I, *Asp*718 and *Hpa*II. The digest patterns of M3 and Ro were different. The *Rsa*I cleavage of Apr differed from all the others, while its *Asp*718 and *Hpa*II cleavage patterns were as in M1, M2 and Pe. The position of the restriction sites along the amplified fragment of each virus isolate is shown in Fig. 2. On the basis of RFLP analysis, the virus isolates were divided into three main groups: (i) M1, M2 and Pe, (ii) M3 and Ro and (iii) Apr. We were unable, however, to distinguish individual members of the group by this method.

Nucleotide sequence analysis

In order to differentiate further between the closely related isolates, the nucleotide sequence of the PCR products was determined (Fig. 3). An additional repeated six-nucleotides-long sequence was found in the amplified products of M3 and Ro at positions 151–156 of RNA 3. This addition resulted in two corresponding extra amino acids arginine (N) and asparagine (R) in the putative polypeptide of the virus coat protein (Fig. 4). These isolates were, therefore, assigned to group PV32, which was previously described by Aparicio *et al*. (1999). Positive identification of the members belonging to that group can be made only by nucleotide sequence analysis. These six repeated extra nucleotides are clearly identified as strain-specific; however, other minor nucleotide modifications were not so clearly defined. Therefore, the differentiation among individual isolates cannot be made solely on the basis of sequence data. TCP was used in an attempt to secure better differentiation.

Transcript conformation polymorphism

Single-strand transcripts of M1 and M2 (which have 99% similarity) (Table 1) copied from the 3'-end slightly differed in their electrophoretic mobility (Fig. 5A). The mobility of the Pe isolate was the same as that of M2, and both had 98% similarity (Table 1). The M3 and Ro transcripts both contain the six extra nucleotides (Fig. 3) and have the same restriction patterns (Fig. 1), and were clear-

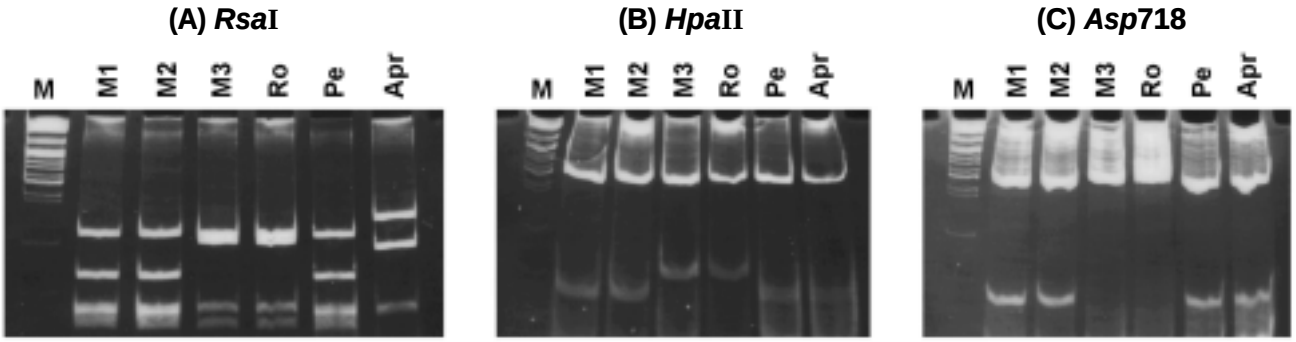

Fig. 1. Differentiation among *Prunus necrotic ringspot virus* isolates by restriction endonuclease cleavage of PCR products. PCR products amplified from total RNA extracted from isolates M1, M2, Pe, M3, Ro and Apr were cleaved with *Rsa*I (A), *Hpa*II (B) and *Asp*718 (C). Digests were fractionated in 6% PAG and stained with ethidium bromide. M, DNA size marker III (Boehringer, Mannheim, Germany).

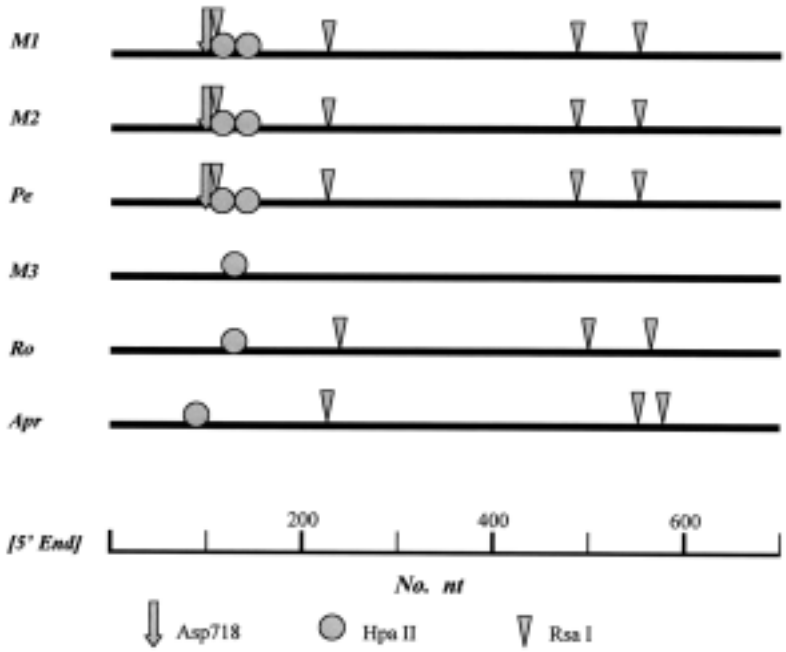

Fig. 2. Restriction map of the PCR products of *Prunus necrotic ringspot virus* isolates; M1, M2, Pe, M3, Ro and Apr. *No. nt*: number of nucleotides.

```
M1      GAGCTCTGGTCCCACTCAGGGCTCAACAGAGGGCTGCGAATAACCC......GAATAGAA  164
M2      ----------------------------------------------......--------
Pe      ----------------------------------------------......--------
M3      ------------------------a-----------------gaatag------g-
Ro      -----a-----------------------a-------------g---gaatag--------
Apr     at-----------------a--------a-----------------......--------

M1      ACCCGAATAGGGCTTCGAGTGGTACCGGACCAGTGGTCCGACCACAACCGGTCGTGAAGA  224
M2      ------------------------------------------------------------
Pe      ----------------------gta-c-ga------------------------------
M3      ------------t----g-c----ta-----t-c---------g----------------
Ro      ------------t------c----ta-----t-c---------g----------------
Apr     -----gtg----t-----a--t-gta-----t-ca--------ca----a----------
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Fig. 3. Nucleotide sequence comparison. Nucleotide (nt) sequences of PCR products derived from the six *Prunus necrotic ringspot virus* isolates were aligned. Identical nucleotides are marked by a dash, modified ones by small letters, deletions by dots, and bold letters mark duplicated nucleotides.

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M1      ALVPLRAQQRAANNP..NRNPNRASSGTGPVVRPQPVVKTTWTVRGPNVPPRIPKGFVAHNH  84
M2      ---------------..------------------------------------------
Pe      ---------------..----------yrt----------i--------------------
M3      ---------------nr------v-g-i--a-------------------------y-----
Ro      -m-----------s-nr------v---i--a-------------------------y-----
Apr     ---------------..----v-v-nvv--a---k-------------------------s-
```

Fig. 4. Amino acid alignment. Multiple alignment of the putative coat protein sequences of the *Prunus necrotic ringspot virus* isolates M1, M2, M3, Pe, Ro and Apr translated from the nucleotide sequences shown in Fig. 3.

ly distinguished by electrophoretic migration in gel. Single-stranded-TCP thus enabled the differentiation of the isolates, which had been impossible with either RFLP or sequence analysis alone. The transcripts initiated from the 5'-end, on the other hand, though they differentiated the Apr isolate from all the others, failed to differentiate between M1, Ro, M3 and Pe (Fig. 5B). These results further demonstrate the unpredictable nature of TCP.

Electrophoretic migration of double-stranded RNA made by annealing a reference transcript of one of the isolates (M1) to complementary transcripts of the other isolates (Fig. 5C) resulted in M1, Pe and M2 duplexes which migrated for about the same distance and the M3 and Ro transcripts

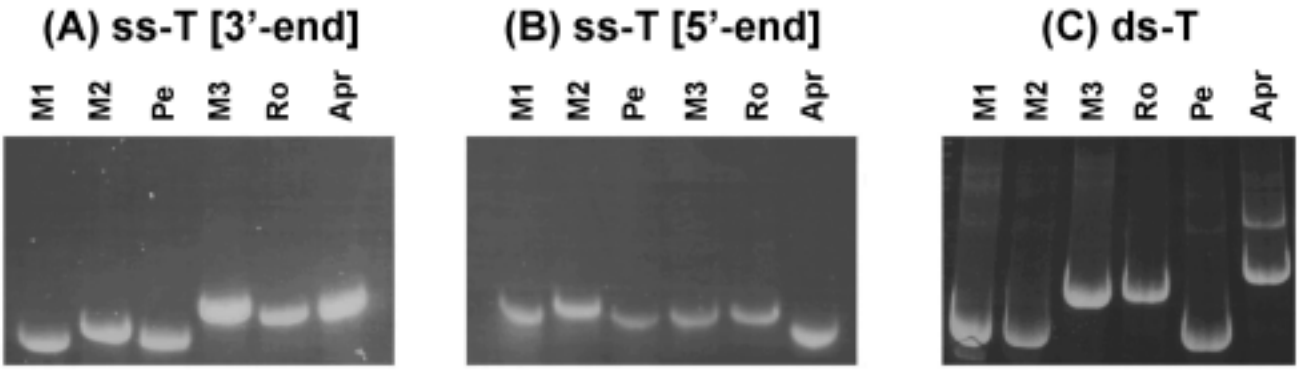

Fig. 5. Single-stranded Transcript Conformation Polymorphism (TCP) of *Prunus necrotic ringspot virus* (PNRSV). RNA transcripts copied from the 3'**(A)** or the 5'**(B)**-end were made from PCR products of six PNRSV isolates, M1, M2, Pe, M3, Ro and Apr, and analyzed by PAGE. **(C)** Differentiation of isolates by ds-TCP. Transcript of isolate M1 at one orientation (transcription with the T7 promoter on the 3'-end of the PCR products) was used as a reference. It was separately annealed to opposite oriented transcripts (copied from a T7 promoter on the 5'-end) of isolates M1, M2, Pe, M3, Ro and Apr to form RNA duplexes which were analyzed by PAGE.

Table 1. Percentage of nucleotide sequence identity. The nucleotide sequence (about 600 base pairs long) of the amplified PCR products derived from the *Prunus necrotic ringspot virus* isolates M1, M2, M3, Pe, Ro and Apr were compared and % similarity between them was determined.

Nucleotide Sequence Identity(%)	***M2***	***Pe***	***M3***	***Ro***	***Apr***
M1	99	98	95	96	84
	M2	98	94	95	84
		Pe	93	94	83
			M3	98	85
				Ro	86

which also migrated an equal distance. These two types of isolates each belonged to a specific virus group (Fig. 1 and Table 1). The duplex of Apr differed and belonged to a separate group. Hence ds-TCP was useful in the initial sorting of virus isolates into groups. However, members within each group (M1, M2 and Pe; and M3 and Ro) could not be distinguished from one another by the electrophoresis of transcript duplexes.

Discussion

The applicability of TCP in combination with other molecular methods for differentiation among virus strains was evaluated. All the isolates were initially characterized by the more-established RFLP and nucleotide sequencing methods. RFLP of PCR products was found to be convenient as a means of dividing virus isolates into groups at an initial stage (Fig. 1), provided the strain-specific restriction sites were well characterized. This method however, was limited in its ability to detect most other nucleotide variations. It identified only nucleotide transitions found within the short recognition sequence of a particular restriction endonuclease (e.g. differentiation among M2 and M3 in Fig. 1 and 2) but did not detect most other modifications residing outside that sequence (see digest of M1, M2 and Pe in Fig. 1)

Nucleotide sequence analysis is essential to detect well-defined strain-specific repeated sequences in RNA3, as shown with M3 and Ro (Fig. 2). It should be noted that the addition of these extra six nucleotides, which was repeated in M3 and Ro, was correlated with the absence of the *Asp*718 site and with a shift in the position of the *Hpa*II site (Fig. 1 and 2). These restriction sites may serve as convenient molecular markers, avoiding sequence analysis. However, most minor nucleotide modifications were not well characterized, and therefore were not sufficiently reliable to differentiate the virus isolates. TCP is generally believed to be affected by multiple nucleotide modifications within the RNA molecule (Sarkar *et al*., 1992; Rosner *et al*., 1998). In this report we have demonstrated that ss-TCP distinguishes closely related virus isolates (as judged by the degree of sequence similarity among them, see % similarity in Table 1). Transcripts of M1 and M2 were distinguished by PAGE as were M3 and Ro (Fig. 5A) which otherwise could not be distinguished by either RFLP (Fig. 1) or sequence analysis (Fig. 3). M1 and Pe seemed to be identical by all tested criteria. However, single-stranded-TCP is an empirical procedure that does not follow strict rules and is almost totally unpredictable in nature. Therefore, it cannot be used to group virus isolates. Instead, this could be achieved by applying ds-TCP, as shown in Fig. 5C. Migration of RNA duplexes in gel was correlated with their classification in groups by RFLP or nucleotide sequence analysis. It is concluded that virus strain differentiation cannot solely rely on any one of the methods described above, but rather needs a combination of all three, each complementing the other at a different level of analysis. Double-stranded-TCP or RFLP could be used for the initial division of virus isolates into major groups. Nucleotide sequence analysis is essential in assigning virus isolates containing strain-specific sequences, and ss-TCP helps in distinguishing between closely related virus isolates.

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