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The Application of RNA Transcript Conformation Polymorphism in Resolving Mixed Infection of PVY Isolates

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A method based on RNA-transcript conformation polymorphism (TCP) was tested for detection of two PVY isolates in a mixed infection. Differences in electrophoretic mobility of RNA transcripts copied from PCR products of each virus isolate enabled the distinction between the two virus isolates in a mixed infection. The identities of the RNA transcripts and hence of the infecting virus isolates were determined by annealing to reference oligonucleotides containing unique strain-specific sequences visualized by retardation of transcript mobility in gel. The ratio at which both virus isolates could be detected was as low as 1:10. The suitability of this procedure for the study of mixed virus infections is discussed.

Keywords **:** *Potato virus* Y, rSSCP, strain differentiation, transcript conformation polymorphism

Resolving closely related individual virus isolates in a mixed infection is the subject of the present communication. *Potato virus Y* (PVY) belongs to the *Potyviridae* family, the largest and economically most important group of plant viruses (Barnett, 1992). Among crop plants it infects mainly potato, tomato and pepper (De Bokx and Huttinga, 1981). The virus model system used in this study was a mixed infection of two PVY isolates. PVY isolates in potato have been divided into three main groups: PVY^{O}, PVY^{N} and PVY^{C}. The first two are the commonest, and they can be serologically differentiated, however, individual members within each group are not always distinguished serologically from one another (see, e.g., PVY^{NTN} in van den Heuvel et al., 1994), and other molecular methods have to be applied. Tordo et al., (1995) demonstrated that the 5'-end region of the PVY genome is highly variable and can be used in the classification of virus isolates. Several molecular methods based on the nucleotide sequence at the 5'-end of the virus genome have been applied to the differentiation of PVY isolates (Singh and Singh, 1996), restriction endonuclease cleavage (Glais et al., 1996; Rosner and Maslenin, 1999a) and PCR amplification (Weidemann and Maiss, 1996). We have previously applied a different approach based on single-stranded conformation polymorphism (rSSCP) of RNA transcripts copied from PCR products (Rosner et al., 1998; Rosner and Maslenin, 1999b); Differences in the nucleotide sequences of RNA transcripts cause conformational changes in the RNA molecules which, in turn, affect their electrophoretic mobility in gel. Virus isolates could be differentiated by using this method termed by us "Transcript Conformation Polymorphism, TCP" (Rosner et al., 1998). We have further demonstrated that a short oligonucleotide sequence derived from a particular virus strain specifically annealed to its corresponding RNA transcript, and thereby cause retardation of transcript mobility in gel (Rosner and Maslenin, 2001). The "tagged" RNA molecules could thus be identified (Rosner and Maslenin, 2003).

Detection of individual virus strain in a mixed infection of closely related virus strains calls for a more complex type of analysis, as these isolates may share identical serological properties and nucleotide sequence homologies. The various methods used for strain differentiation could be applied for resolving mixed virus infection. In the present communication we report on the application of RNA transcript conformation polymorphism in combination with the annealing of transcripts to strain-specific oligonucleotides in the analysis of a mixed infection of two PVY isolates.

Materials and methods

Virus isolates. A potato tuber necrosis strain of $PVY\text{-}Y^{NTN}$-H (Nicola, Hessen) was kindly given to us by Dr. Weidemann (Braunschweig, Germany). Three virus isolates were provided by Dr. Camille Kerlan (INRA, France): PVY^{N}-W (N242) (accession number: AF248499, Glais et al., 2002), isolated in Northern France; PVY^{N}-B203, isolated in Brittany, France (Glais et al., 2002); and PVY^{N} - N605 (accession number: X97895) a Swiss isolate. PVY^{N} RB, an N-strain field isolate from Scotland was supplied to us by Dr. Ian Barker (CSL, York, UK). PVY- 52 (N-strain) and DL (O-strain) are two local field isolates from Israel.
RNA extraction. Total RNA extraction from plant tissue

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(100 mg of leaves) was carried out by means of the lithium method (Spiegel et al., 1996).

PCR primers. The following primers were used in PCR amplification of the 5-end PVY genome:

(1) 5'-AAAACAACTCAATACA-3'
(forward, position 1-20)

(2) 5'-AGTACCGTTCTTGAGCACAC-3'
(reverse, position 386-366)

The T7 RNA polymerase promoter sequence (5'-AATTTAATACGACTCACTATA-3', see Rosner et al., 1998) was attached to the 5'-end of this primer.

RT-PCR. The protocol for the one-tube RT-PCR reaction was according to Rosner et al. (1998).

Transcription. A 2-µl sample of PCR products containing the T7 RNA-promoter sequence was incubated for 1 h at 37°C together with 4 µl of transcription buffer × 5 (supplied with the enzyme), 4 µl of rNTP mixture (2.5 mM each), 2 µl DTT (0.1 M), 1 µl of RNasin (5 U/µl; Promega), 6 µl of water (total volume 20 µl) and 1 µl of T7 RNA polymerase (50 U/µl; Biolabs).

Annealing of oligonucleotides. The following oligonucleotides sequences were used:

(I) 5'-ATTTCGATCTTCATCAAACA-3'
(position 91-111)

(II) 5'-TCCAACCAATTTCAGATCCTC-3'
(position 162-185)

The sequences of oligonucleotides-I and -II were derived from PVY^{NTN} and the N-strain respectively (GenBank accession numbers M95491 and D00441, respectively; see also Rosner and Maslenin, 2000b, 2003) were designed to be complementary to the RNA transcripts.

The tested oligonucleotide (200 ng) was added to a freshly prepared RNA transcript (2 µl) copied from the PCR products, in a mixture containing buffer B (Roche Diagnostics, Mannheim, Germany) in a total volume of 10 µl. Annealing was carried out by incubating the reaction mixture for 15 min at 54° and then at room temperature for an additional 15 min prior to application to the gel.

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 (5 µl), and fractionation was carried out by electrophoresis (applied voltage: 50 V) in a 10 × 8 cm polyacrylamide gel (PAG) in 40 mM Tris-acetate, pH 8.0; 1 mM EDTA (TAE) buffer, at room temperature in a vertical minigel apparatus (BioRad Laboratories, Hercules, U.S.A.) or in an agarose gel.

Restriction endonuclease analysis. Aliquots (6 µl) of the amplified PCR products were digested in the presence of appropriate buffer by adding the tested restriction endonuclease (0.5 U) in a total volume of 15 µl. The reaction was incubated at the recommended temperature for 2 h and fractionated by PAGE.

Results

TCP of PVY isolates. PCR products of six PVY isolates were transcribed into single-stranded RNA and fractionated in gel (Fig. 1). While transcripts of three isolates - 52, 203 and W - were non-polymorphic, those of isolates 605, RB and DL differed in electrophoretic mobility, among themselves and from the first three isolates. The fastest moving transcript was that of isolate RB.

Detection of transcripts in mixed infection. PVY isolates 52 and RB served as a model system for the study of mixed infections, as each belonged to one of the two major groups of PVY isolates that infect potato, classified by their 5-end UTR sequences and RNA transcripts of these two virus isolates markedly differed in their electrophoretic mobility in gel (Fig. 1).

PCR products of total RNA extracted from plants dually infected with PVY strains; 52 and RB were indistinguishable in size (Fig. 2A). Copying these products yielded two distinct RNA transcripts; one with a slower mobility corresponding to the mobility of isolate 52 transcript and the other, a faster moving band similar to that of RB isolate (Fig. 2B). Thus, the two virus isolates could be clearly resolved in the mixed infection.

Identification of transcripts by annealing to reference oligonucleotides. Oligonucleotides-I and -II, which contained sequences unique to isolates 52 and RB, respectively, were each annealed to RNA transcripts of the two virus isolates (Fig. 3A). Oligo-I caused an upward shift in the electrophoretic migration of the 52-transcript, but did not have any effect on the mobility of the RB-transcript. Oligo-II, on the other hand, had a slight gel retardation affect on

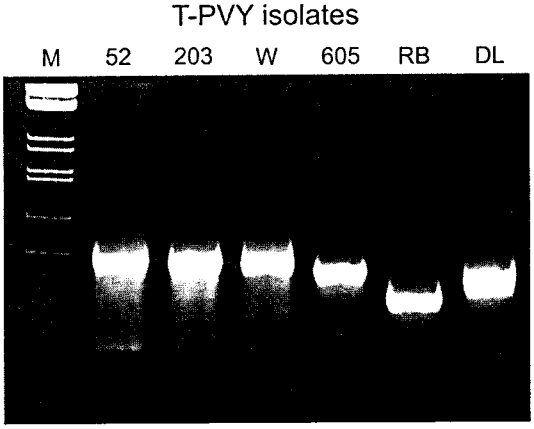

Fig. 1. Mobility of RNA transcripts of PVY isolates. PCR products made from total RNA of tobacco plants infected with PVY isolates 52, 203, W, 605, RB and DL were used as templates for transcription of RNA which was fractionated by PAGE and stained with ethidium bromide. M, size marker of EcoR I/Hind III cleaved Lambda DNA.

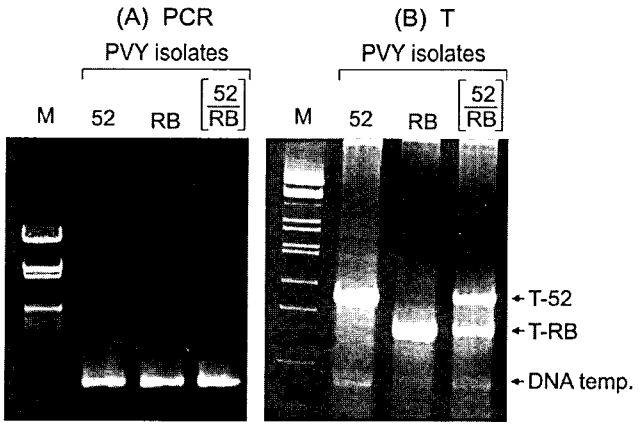

Fig. 2. Detection of PVY isolates in mixed infection by TCP. Panal (A) Total RNA of tobacco plants separately infected with isolates 52 and RB and from plants mixed infected with the two virus isolates [52/RB], served as templates for amplification by PCR. Panal (B) The PCR products in (A) (see arrow "DNA temp.") were copied to RNA and the transcripts (T) were fractionation in gel as described in the legend to Fig. 1.

the RB-transcript and had no effect on the mobility of the 52-transcript. The shift in electrophoretic mobility can thus be applied to the identification of these virus isolates in mixed infection. Oligo-I and -II were separately annealed to RNA transcripts prepared from mixed infection of isolates 52 and RB (Fig. 3B). Oligo-I (52-specific) caused a shift in migration of the upper transcript band, whereas oligo-II (RB-specific) slowed the migration of the lower one thus identifying these transcripts as belonging to isolates 52 and RB, respectively.

Detection of virus ratios in mixed infection. Linked PCR-transcription of mixtures of RNA which contained constant concentrations of total plant RNA infected with isolate 52 and a series of decreasing amounts of RNA from RB-infected plants (Fig. 4A), revealed that RB-related RNA could be detected at a low ratio of 52/RB of 1:10.

The minimum amount of DNA template required for obtaining visible amounts of transcripts was 6 ng (1/100 dilution), as determined by transcription (T) of serial dilutions of PCR products (Fig. 4B).

Study of mixed infection by restriction endonuclease cleavage analysis. Mixed infection of isolates 52 and RB could also be resolved by restriction endonuclease cleavage of the PCR products, by using *Nco* I and *Bgl* II (Fig. 5), (see Rosner and Maslenin, 2000a); *Nco* I uniquely cleavaged products of isolate 52, to yield two bands, whereas products of the RB isolate remained uncut. *Bgl* II, on the other hand, digested only the RB products (Fig. 5A). Cleavage of PCR products of mixed infection (Fig. 5B) by *Nco* I and *Bgl* II produced each similar bands in addition to an uncleaved one which corresponded to the RB isolate in the *Nco* I digest and to the 52 isolate in the *Bgl* II cleavage. It should be noted that restriction patterns naturally contained more than one band for each virus isolate (Fig. 5), whereas in the TCP analysis a single clear transcript band was observed for each virus isolate (Fig. 2).

Discussion

The resolution of individual virus isolates in a mixed infection of closely related viruses is based on procedures

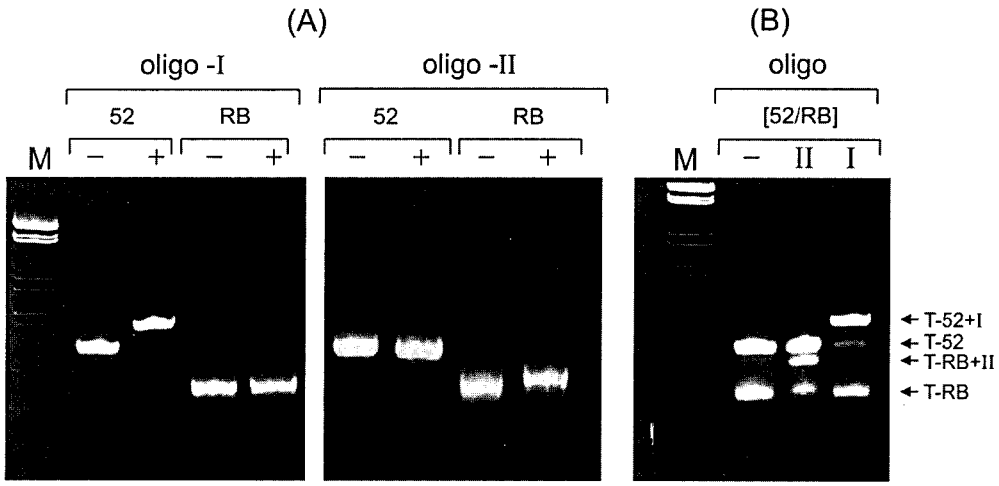

Fig. 3. Annealing of RNA transcripts to reference oligonucleotides. Panal (A) Oligonucleotides (oligo)-I and II were each annealed to RNA transcripts of isolates 52 and RB (+), followed by gel fractionation. Control of transcripts without the added oligonucleotides (−) were included. Panal (B) Oligo-I and II were annealed separately (+) to RNA-transcripts copied from PCR products of [52/RB]-mixed infected tobacco plants. The arrows mark the positions of the 52 and RB-transcripts (T-52 and T-RBrespectively) and with the added oligonucleotides-I and II (+I and +II).

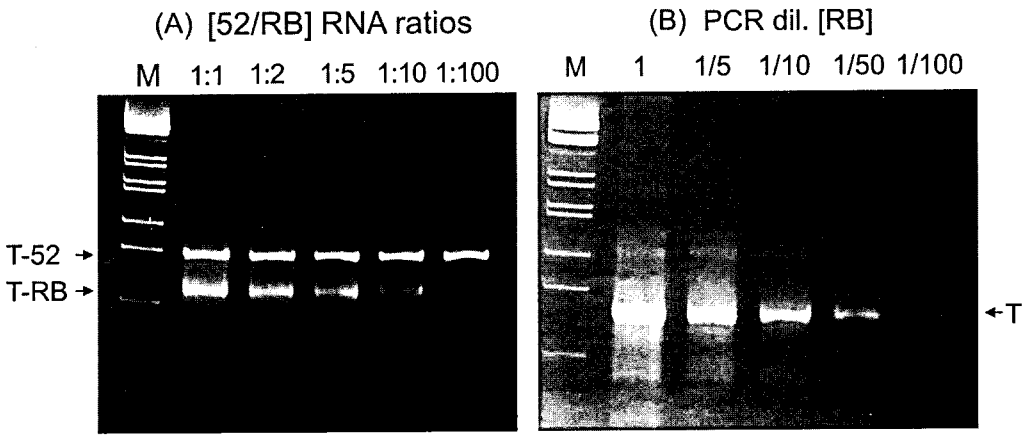

Fig. 4. Detection of various ratios of PVY isolates in mixed infection. Panal (A) Total RNA of plants infected with either isolate 52 or RB were mixed as follows: constant undiluted concentration of 52-RNA was mixed with equal volumes of decreasing amounts of RB-RNA: 1:1, 1:2, 1:5, 1:10 and 1:100 and the mixtures were subjected to a linked PCR-transcription reaction yielding 52 and RB-transcripts (T-see arrows). The RNA transcripts were fractionated in gel. Panal (B) PCR products made from RB-infected plants (0.64 μg/μl) were serially diluted; c-concentrated, 1:5, 1:10, 1:50 and 1:100. The diluted mixtures served as templates for transcription of RNA (T) fractionated by gel electrophoresis.

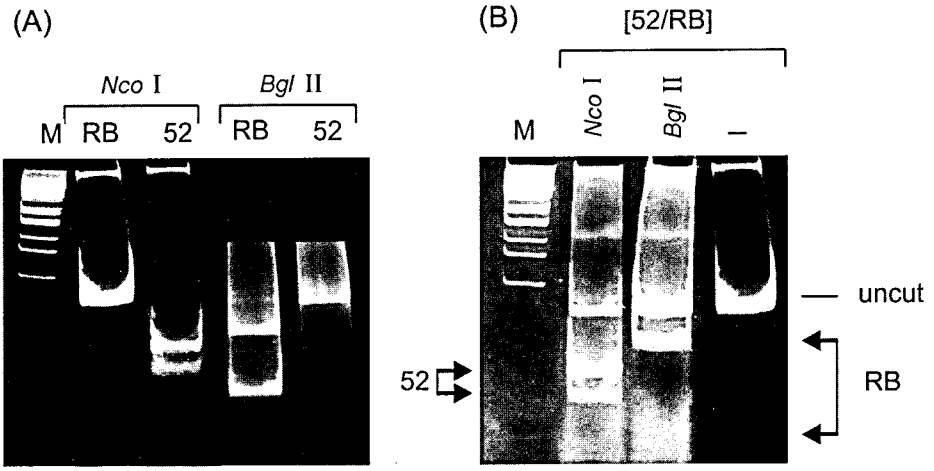

Fig. 5. Restriction endonuclease analysis of PCR products originated from mixed-infected plants. PCR products amplified from plants infected separately with isolates 52 and RB (A) or both together [52/RB] (B) were cleaved with *Nco* I and *Bgl* II. Uncleaved sample(-)was included. The digests were fractionated in gel by electrophoresis.

applied to the differentiation among virus strains. Serological methods (ELISA) based on the use of polyclonal and monoclonal antibodies (Spiegel et al., 1999), PCR (Weidemann and Maiss, 1996), RFLP (Glais et al., 1996), DNA-SSCP (Magome et al., 1999) and RNA-rSSCP (TCP) (Rosner et al., 1998, Rosner and Maslenin, 1999b) were applied in several virus systems. While the serological methods are limited for detection of differences within a single gene product - the virus coat protein, molecular methods which are based on variations at the nucleotide sequence level, screen for a much wider range of modifications - covering the whole virus genome. We have previously reported on the detection of two PVY isolates in mixed infection by restriction endonuclease analysis (Rosner and Maslenin, 1999a) and by annealing to reference RNA transcripts (Rosner and Maslenin, 2003). In the present study we applied RNA-rSSCP (TCP) to the detection of PVY isolates in a mixed infection by studying the differences in electrophoretic mobility of RNA transcripts copied from PCR products (Fig. 2). The annealing of RNA transcripts to strain-specific oligonucleotide reference sequences (visualized by retardation of transcript mobility in gel, Fig. 3) enabled the identification of transcripts and, hence, of the corresponding infecting virus isolates in the mixed infection.

It should be noted that the TCP method can be applied only for the analysis of virus isolates that exhibit transcript conformation polymorphism; e.g., transcripts of isolates

605, RB, and DL differ in electrophoretic mobility, both among themselves and relative to the other three isolates that were tested (52, 203 and W) whose transcript mobilities were identical and could not be distinguished (Fig. 1). The *Nco* I/*Bgl* II restriction cleavage also does not differentiate among isolates 52, 203 and W and isolate RB from DL (not shown). The TCP method, on the other hand, distinguish between the last two isolates including isolate 605 (Fig. 1). The TCP procedure has two advantages: (a) It is simpler to interpret than RFLP analysis, since it yields a single clear band of RNA transcript corresponding to each virus isolate in the mixture (Fig. 2), whereas cleavage of PCR products by restriction endonucleases generates several bands, and the pattern obtained, therefore, is somewhat more complex (Fig. 5). (b) The identity of the transcript can be determined by annealing to reference oligonucleotides (provided unique strain specific sequences are available) (Fig. 3). The TCP method is thus suitable for the study of mixed infection of virus isolates that fulfill the above-mentioned criteria.

Acknowledgment

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