Interference in PCR: Transcription Amplifications of Mixed PVY Isolates

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Abstract

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Interference in PCR and transcription amplifications by mixed virus templates is described. The yields of PCR amplification of closely related PVY isolates in a mixture were lower than those of the separate amplification of each individual virus strain, i.e. the two virus templates mutually interfered in their amplification process. When PCR products of mixed PVY isolates served as templates for transcription, RNA synthesis was also inhibited. This interference could be avoided by applying strain-specific primers in the separate amplification of each target sequence in the mixture. The use of strain-specific primers in PCR amplification of a mixed virus infection thus enabled detection of each virus isolate without interference.

Keywords: potato virus Y; rSSCP; RFLP; mixed infection; interference; conformation polymorphism; PCR

PVY isolates in potato are divided into three main groups: PVY^O, PVY^N and PVY^C (De Bokx & Huttinga 1981). The first two are the most common, and can be serologically differentiated. However, individual members within each group were not readily distinguished (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 useful 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 for the differentiation of PVY isolates: nucleotide sequence analysis (Singh & Singh 1996), restriction endonuclease cleavage (Glais *et al.* 1996; Rosner & Maslenin 1999a), PCR amplification (Weidemann & Maiss 1996) and single-stranded conformation polymorphism (rSSCP) of RNA transcripts copied from PCR products (Rosner & Maslenin 1999b).

In the present communication we report on interference in PCR amplification and transcription of two PVY isolates in a mixed infection. We found that this interference can be avoided by applying strain-specific primers for the separate amplification of each target sequence in a mixed virus infection.

MATERIALS AND METHODS

Virus isolates. PVY^N-RB, an N-strain field isolate from Scotland, was supplied to us by Dr. Ian Barker (CSL, York, UK), and PVY-52 (N-strain) is a local isolate from Israel.

RNA extraction. Total RNA extraction from plant tissue (100 mg of leaves) was carried out by means of the lithium method (Spiegel *et al.* 1996).

PCR primers. Universal primers for PCR amplification of the 5'-end PVY genome:

(1) 5'-AATTAAAACAACTCAATACA-3' (forward, position 1–20)

(2) 5'-TGGGCATCAGTCTTGTATCG-3' (reverse, position 391–411)

The sequences were derived from the full-length PVY sequence (accession number D00441) described by Robaglia *et al.* (1989).

The T7-RNA polymerase promoter sequence (5'-AATTTAATACGACTCACTATA-3', Rosner & Maslenin 2001) was added to the 5'-end of the above two primers as indicated.

Strain specific primers were derived from sequence comparison in Rosner and Maslenin (2003):

(I) 5'-CTGAAATTGGTTGGAAGTGA-3'
     [RB specific] (reverse, position 158–178)
(II) 5'-GTAAATTGCAGAAGATCATC-3'
     [52-specific] (forward, position 168–187)

For specific amplification of the RB isolate sequence the primer pair (I) and (1) was applied, and for the 52 isolate a combination of primers (II) and (2). The sizes of the PCR products were 178 and 243 nucleotides, respectively.

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 (2.5mM each), 2 µl DTT (0.1M), 1 µl of RNasin (5 U/µl; Promega, Madison, U.S.A.), 6 µl of water (total volume 20 µl) and 1 µl of T7 RNA polymerase (50 U/µl; BioRad Laboratories, Hercules, U.S.A.).

Restriction endonuclease cleavage. About 1 µg of plasmid DNA was digested in the presence of the supplied buffers by Nco I and Bgl II (0.5 U) (Promega, Madison, U.S.A.) in a total volume of 15 µl at 37°C for 2 h.

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 6% polyacrylamide gel (PAG) in 40mM Tris-acetate, pH 8.0; 1mM EDTA (TAE) buffer, at room temperature in a vertical minigel apparatus (BioRad Laboratories, Hercules, U.S.A.) or in 1% agarose gel.

RESULTS

PCR amplification of mixed virus templates

A series of mixtures of two closely related virus isolates, which contained identical amounts of isolate 52 and decreasing concentrations of the RB template, were simultaneously amplified by PCR,
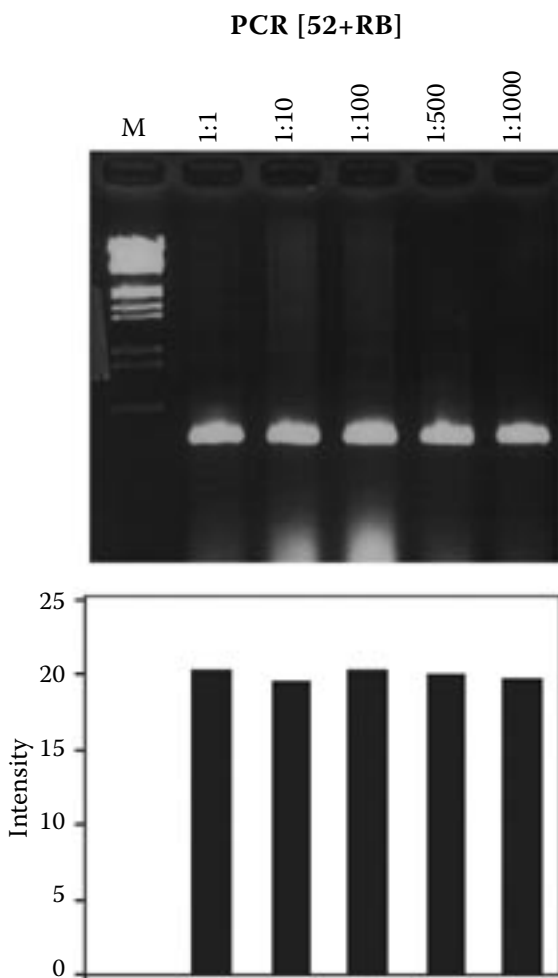

Figure 1. PCR amplification of mixed templates of PVY isolates. Plasmid clones of two PVY isolates: 52 (2.7 µg/µl) and RB (2.6 µg/µl) were mixed in the ratios indicated in the figure, were amplified by PCR with the universal primers 1 and 2-T7-promoter (see Materials and Methods) and the products were fractionated in agarose gel. The intensity of each band in the gel (minus background), was quantitatively determined with the PC Stand Alone program and the data were plotted as columns by using Microsoft Excel. M- Marker, 3 of EcoR I/Hind III cleaved Lambda DNA (Fermentas Life Sciences, Lithuania)

with universal primers (Figure 1). The resulting products were indistinguishable in size and had about the same yields as determined by measuring the intensity of each band in the gel (see corresponding columns in the graph of Figure 1).

Restriction enzyme analysis

The products of the mixed virus isolates were resolved by strain-specific restriction endonuclease cleavage (Figure 2). Digestion with Nco I (which uniquely cleaved PCR products of the 52 isolate) revealed that a reduction in the concentration of the RB template (and hence in its amplified products) to one-hundredth was correlated with a threefold increase in the amounts of fragments related to the 52 isolate (see graph in Figure 2A). Similar results were obtained when Bgl II, which cleaved only the RB products, was applied (see curve in Figure 2B). It seems, therefore, that the two virus templates mutually interfere in their amplification processes.

Interference in transcription of mixed PCR products

In a different approach, the amplified products of mixed virus strains were resolved by rSSCP. PCR products of a mixture of RB and 52 templates were transcribed and the RNA was analysed in gel (Figure 3). The intensities of the RNA bands of each virus isolate were 2–2.5 times higher than those of the bands obtained by the simultaneous transcription of both in a mixture (see relative intensities in the graph of Figure 3). A 1:1000 dilution of the RB template, was correlated with a 2.5 times increase in the intensity of RNA bands of the 52 isolate. It seems that the DNA template of one isolate suppressed transcription of the other.

PCR amplification of virus isolates by using strain-specific primers

In order to avoid interference, strain-specific primers were tested in multiplex PCR. In a search

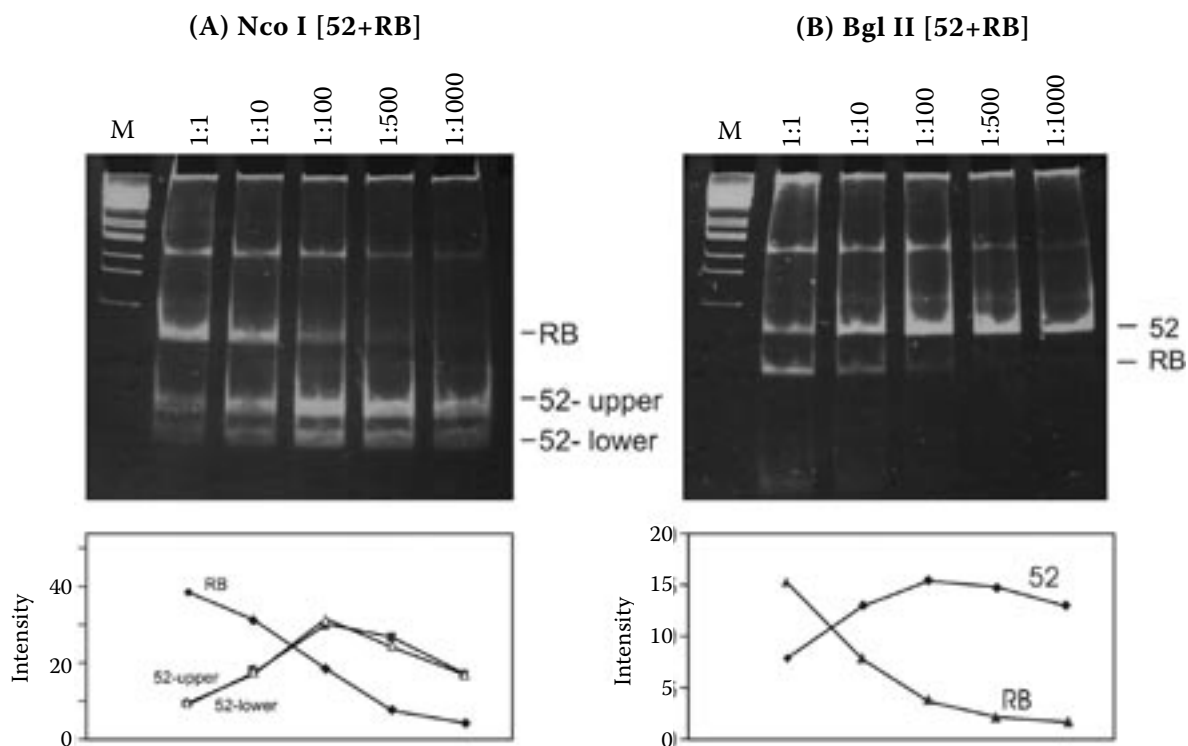**(A) Nco I [52+RB]**   **(B) Bgl II [52+RB]**

Figure 2. Resolution of PCR products of individual virus isolates by strain-specific restriction cleavage. Virus concentrations were determined serologically by ELISA. Equal amounts of each virus strain (about 1 ELISA unit): RB and 52 were used for the preparation of RNA templates used in the amplification of mixed strains. PCR products of mixed templates [52+RB] in ratios shown in Figure 1 were cleaved with Nco I (A) and Bgl II (B) and the digests were fractionated by PAGE. Fragments belonging to each isolate are indicated. The relative intensity of bands of each DNA fragment was determined as described in the legend to Figure 1 and the data were plotted in the accompanying graph. M- Marker, 3 of EcoR I/Hind III cleaved Lambda DNA (Fermentas Life Sciences, Lithuania)
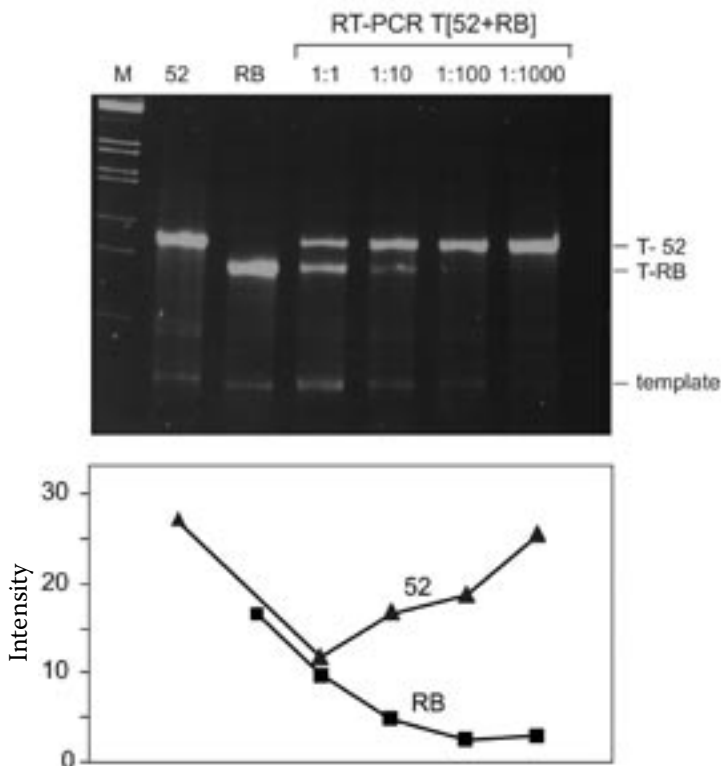
Figure 3. Interference in transcription of mixed virus templates. Equal amounts of viral RNA of the 52 and RB isolates (as determined by ELISA) were amplified separately by RT-PCR with the universal primers 1 and 2-T7-promoter and in mixtures at various 52:RB ratios, with a constant concentration of 52 and a series of decreasing RB concentrations, as indicated in the figure. The RNA transcripts (T) were analysed by PAGE. The positions of transcript bands corresponding to the 52 and RB isolates are indicated. The intensity of each band was determined and plotted. M – Marker, 3 of EcoR I/Hind III cleaved Lambda DNA (Fermentas Life Sciences, Lithuania)

for strain-specific primers (Rosner & Maslenin 2003), primer pairs that exhibited the highest degree of specificity for the RB (I) and 52 (II) isolates were chosen (see Materials and Methods). It was found (Figure 4) that the pairs of strain-specific primers amplified only their corresponding virus
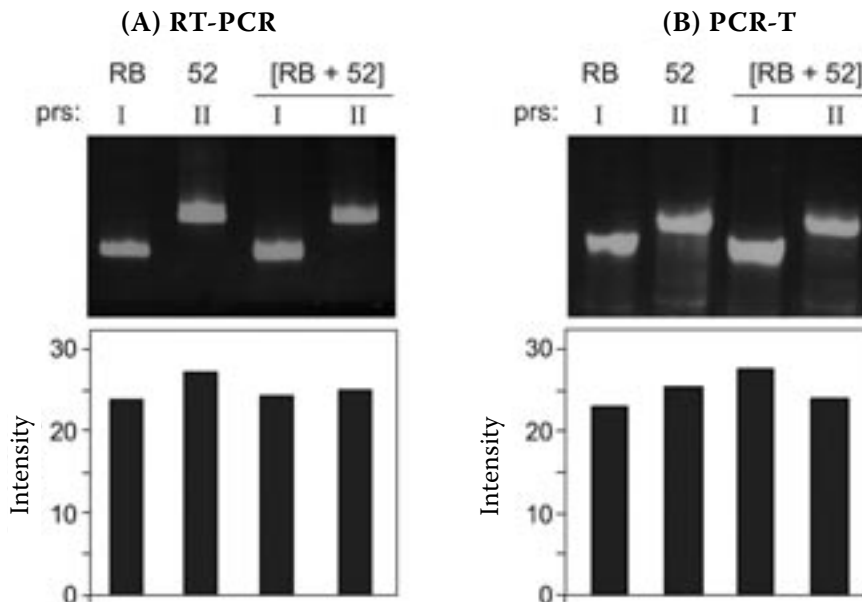

Figure 4. The application of strain-specific primers in PCR amplification. Samples of total RNA from plants individually infected with the RB or the 52 isolate, or with a mixture of [RB+52] were amplified by RT-PCR with pairs of strain-specific primers: (I) – (RB-specific)/1-T7-promoter (universal) and (II) – (52-specific)/2-T7-promoter (universal). The PCR products were fractionated by agarose gel electrophoresis (A); or were transcribed and the RNA-transcripts were similarly fractionated (B). The relative intensities of bands were determined and the data were plotted as columns under each figure

templates, either in a mixture of [RB+52] or in controls that contained one of the individual virus isolates [RB or 52] (Figure 4A). The intensity of all PCR products (see columns in Figure 4A) were about the same, indicating that no interference in the amplification of mixed-virus templates had occurred.

Transcription of PCR products that originated from the mixture of virus isolates ([RB+52] in Figure 4B) also showed no evidence of interference (compare the intensities of transcripts made from the separate and mixed virus templates that were plotted as columns in Figure 4B). It should be noted, though, that in this particular case – in which the PCR products of the two virus isolates already differ in size – the application of transcript conformation polymorphism was of no additional value.

The use of strain-specific primers in PCR amplification of mixed virus infection thus enabled the detection of each virus isolate without interference from the other.

DISCUSSION

Two levels of interference were found between the templates of two closely related PVY isolates in linked PCR-transcription amplification (Figures 2 and 3). It seems that there was mutual interference between the two virus templates during the PCR amplification process, so that in a mixture an increase in the concentration of one virus suppressed the amplification of the other. Thus, the amounts of amplified products did not reflect the original ratio between the two infecting virus isolates. The cause of the interference phenomenon is not yet clear. A previous report stated that high concentrations of DNA template reduce amplification yields (Kary *et al.* 1987). It was hypothesised that with a high concentration of target DNA the amplified fragments re-hybridised more readily rather than being annealed to the primer molecules, with the result that the amplification efficiency was reduced. However, in our present system, lowering the concentration of virus templates in the mixture did not affect the interference phenomenon (not shown).

It should be noted that in multiplex PCR amplification of PVY isolates described in Nie and Singh (2003) no interference was observed as in the system described in the present report. This may be explained by the following arguments: In a multiplex system different sets of primers were used in a single mixture to amplify different target sequences. In our studies one set of primers is used to amplify two very similar target templates which have identical binding sites for these primers. It may be speculated that the two templates compete with each other for binding of primers and thus negatively affect their amplification. In a multiplex system, on the other hand, each set of primers has different binding sites on different target sequences so that no competition takes place. This may explain the differences in interference observed between our mixed strains amplification system and the multiplex PCR.

The interference described in the present manuscript and not found by others in a multiplex system using a single reverse primer and a different forward one (Nie & Singh 2002), may be similarly explained. Although one primer is identical, the other is not; the interference phenomenon is thus avoided. It is shown also in this report (Figure 4) that non-identical primers do not to interfere with each others' amplification.

The interference also occurred in transcription of mixed PCR templates (Figure 3). Milligan *et al.* (1987) indicated that 50 nM to 1 μM is the optimal concentration range of DNA template for transcription. In the present study we found that high concentrations of DNA template strongly inhibited transcription. It may be speculated that interference in both PCR and transcription is caused by competition of templates for as yet unidentified limiting factor(s) in these reactions.

It should be noted that PCR as such is not a quantitative measurement (Rosner *et al.* 1992). "Real-Time"-PCR (Roberts *et al.* 2000) enables the quantitative determination of virus concentration, but mutual interference in the amplification of mixed virus templates may still be a problem.

We have demonstrated in the present study that the application of strain-specific primers for the separate amplification of each individual target sequence in the mixture avoids the template interference problem in either PCR or transcription (Figure 4). Therefore, this should be the method of choice wherever strain-specific sequences are to be identified.

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Abstrakt

Rosner A., Maslenin L. (2005): **Interference směsi izolátů PVY v PCR a transkripci**. Plant Protect. Sci., **41**: 125–131.

Byla prokázána interference izolátů virů v PCR a transkripci. Výtěžek PCR blízce příbuzných izolátů *Y* viru bramboru (PVY) byl nižší při jejich amplifikaci ve směsi, než když byly izoláty amplifikovány odděleně. Z toho vyplývá, že templát jednoho viru ovlivňoval ve směsi proces amplifikace druhého viru. Syntéza RNA byla inhibována i v případě, když jako templát pro transkripci sloužily produkty PCR směsi izolátů PVY. Vzájemnému

ovlivnění izolátů virů může být zabráněno použitím kmenově specifických primerů. Kmenově specifické primery umožňují amplifikaci každého izolátu viru ze směsné infekce bez jejich vzájemné interference.

Klíčová slova: *Y* virus bramboru; rSSCP; RFLP; směsná infekce; interference; konformační polymorfismus; PCR

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