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Self-priming on the plant viral RNAs during reverse transcription-PCR

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Summary. – The occurrence of the primer-independent cDNA synthesis during RT-PCR analysis of human and animal RNA viruses has been well documented. Conversely, there is scant knowledge about this event in plant RNA viruses. Here we show that the primer-independent cDNA synthesis occurs in all eight different plant RNA viruses tested in this study, suggesting a common phenomenon for RT-PCR analysis of plant RNA viruses. Additional experiments indicate that the event is likely contributed to by RNA self-priming, and can be effectively reduced or eliminated through increasing temperature of the RT reaction.

Keywords: RT-PCR; primer-independent cDNA synthesis; self-priming; plant RNA virus; RT temperature

RT-PCR is one of the most common methods of molecular biology used to detect and quantify RNA expression levels in both cells and small quantities of tissues. Within this process, RT is the first and fundamental step, in which the RNA template is converted into its cDNA by reverse transcriptase in the presence of exogenous oligonucleotide referred to as primer, providing template DNA for subsequent PCR amplification. The primer-dependent mechanism for RT reaction has been generally accepted over the years. However, during RT-PCR analysis of human and animal RNA viruses, bacterial operons, as well as eukaryotic cellular RNAs, it has been observed that cDNA could be synthesized by reverse transcriptase without the addition of exogenous oligonucleotide (Gunji *et al*., 1994; Lanford *et al*., 1995; Lerat *et al*., 1996; Schoenike *et al*., 1999; Guacucano *et al*., 2000; Peyrefitte *et al*., 2003; Haddad *et al*., 2007; Tuiskunen *et al*., 2010, Moison *et al*., 2011). This completely contradicts the well-accepted mechanism of cDNA synthesis, indicating that RT occurs in a primer-independent manner.

Thus far, several explanations have been proposed to clarify the origin of the primer-independent event. It was postulated that *Taq* polymerase probably possesses the background reverse transcriptase activity that might be active in the PCR step once forward and reverse primers are present, thus leading to the confusing result (Martel *et al*., 2002). However, many lines of evidence supported the possibility that this kind of cDNA synthesis is most likely primed by cellular small nucleic acids (DNA, microRNA, tRNA, etc.) associated with the commercial reverse transcriptase as well as template RNA, or by the thermosTable hairpin structure at the 3'-end of the template RNA, the so called self-priming (Agranovsky, 1992; Gunji *et al*., 1994; Lerat *et al*., 1996; Timofeeva and Skrypina, 2001; Piche and Schernthaner, 2003; Haddad *et al*., 2007; Tuiskunen *et al*., 2010; Moison *et al*., 2011). Regardless, since the primer-independent RT usually contributes to non-specific cDNA synthesis that may interfere with the PCR specificity, thus resulting in misinterpretation of the final experimental data, much effort has been devoted to overcoming this unexpected event (Lerat *et al*., 1996; Peyrefitte *et al*., 2003; Haddad *et al*., 2007; Moison *et al*., 2011). Of the developed strategies, tagged RT primer

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Abbreviations: CGMMV = cucumber green mottle mosaic virus; CMV = cucumber mosaic virus; gRNA = genomic RNA; ORSV = odontoglossum ring-spot virus; PVX = potato virus X; TCV = turnip crinkle virus; TMV U1 = tobacco mosaic virus U1 strain; TNV = tobacco necrosis virus; TRV = tobacco rattle virus

as well as high temperature reverse transcription has been proved to be effective in achieving strand-specific amplification (Peyrefitte *et al*., 2003; Haddad *et al*., 2007; Moison *et al*., 2011). It is worth to note that the two methods rely on different mechanisms. The former allows making the falsely primed cDNA undetecTable rather than to avoid it, whereas the latter maintains the RT reaction at high temperature to minimize the non-specific cDNA synthesis.

Plant RNA viruses are a group of major agricultural pathogens that cause a number of economically important plant diseases worldwide (Reddy *et al*., 2009; Scholthof *et al*., 2011). To mitigate or prevent a viral disease from occurring, the conventional RT-PCR has become the one routine technique to detect the plant RNA viruses due to its sensitivity and ready availability (Thomson and Dietzgen, 1995). So far, however, little attention was paid to the primer-independent cDNA synthesis during plant RNA virus identification or diagnosis, probably because this event has no or little effect on the final result of common RT-PCR used for these purposes. Tobacco mosaic virus (TMV) U1 is one of the most well-known plant RNA viruses that belongs to the genus *Tobamovirus* (Creager *et al*., 1999; Scholthof *et al*., 2011). Recently, we showed that this positive-strand RNA virus, which is known to terminate with 3' tRNA-like structures, possesses a small proportion of genomic RNA (gRNA) bearing polyadenylated tails (Li *et al*., 2014). Regarding the nature of the poly(A) tails, we initially performed RT-PCR with oligo(dT)$_{18}$ as RT primer to characterize these special TMV gRNA molecules. However, the same PCR product could also be produced in the negative control experiment, wherein the RT reaction was carried out without addition of oligo(dT)$_{18}$ as well as any other exogenous primer (data not shown). This unexpected data implied that the primer-independent cDNA synthesis might occurred during RT-PCR detection of TMV gRNA but await further elucidation.

To address this concern, in the present study we performed RT-PCR on TMV gRNA as described below. Total RNA was first extracted from the TMV U1-infected *Nicotiana benthamiana* leaves with Trizol (Invitrogen) according to the manufacturer's instructions and used as a template to perform RT with M-MLV Reverse Transcriptase, RNase H Minus (Promega) as follows: 1 μg of total RNA was denatured at 65°C for 5 min in the presence of 20 pmol TMV-6395-76 (Table 1), a gene-specific primer corresponding to the extreme 3' end of TMV gRNA, or without any primer addition, then was chilled on ice for 2 min; the RT reactions in a total volume of 25 μl containing 0.5 mmol/l of dNTPs, 200 U of M-MLV, 5 μl reaction buffer (5×), 10 U RNase inhibitor (Promega) were subsequently incubated at 42°C for 1 hr, followed by a heating step at 95°C for 10 min to inactivate RT enzyme. The amplification of the cDNA product as a template was carried out immediately using LA Taq (Takara) in a reaction containing 1 μl cDNA in a 20 μl reaction mix containing 10 pmol of each of the primers TMV-6023-44 and

Table 1. Oligonucleotides used in this study[a]

Oligo name	Organism	Application	Direction	Primer sequence (5'-3')
TMV-6023-44	TMV	PCR	Forward	GACTGCCGAAACGTTAGATGCT
TMV-6395-76	TMV	RT/PCR	Reverse	TGGGCCCCTACCGGGGGTAA
CGMMV-5763-83	CGMMV	PCR	Forward	ATGGCTTACAATCCGATCACA
CGMMV-6424-05	CGMMV	RT/PCR	Reverse	TGGGCCCCTACCCGGGGAAA
ORSV-6065-85	ORSV	PCR	Forward	CAACTCGTAGAGTTGATGATG
ORSV-6618-597	ORSV	RT/PCR	Reverse	TGGGCCTCTACCCGAGGTAA
CMV1-2698-717	CMV(RNA1)	PCR	Forward	CACGAAATGGGTTTCTCAAT
CMV1-3357-38	CMV(RNA1)	RT-PCR	Reverse	TGGTCTCCTTTTAGAGACCC
TRV1-6177-97	TRV(RNA1)	PCR	Forward	GGTCATGCTAACAAATTGCGA
TRV1-6791-72	TRV(RNA1)	RT/PCR	Reverse	GGGCGTAATAACGCTTACGT
TCV-3564-84	TCV	PCR	Forward	GAGCACGATTGTCATTTTCTC
TCV-4050-28	TCV	RT/PCR	Reverse	GGGCAGGCCCCCCCCCCGCGCGA
TNV-3021-41	TNV	PCR	Forward	GTGAGCTCATCTACATACCTA
TNV-3682-63	TNV	RT/PCR	Reverse	GGGGTGGGGCAAAAGCCCCT
PVX5866-85	PVX	PCR	Forward	ACAGACACTATGGCACAGGC
PVX6435-13	PVX	RT/PCR	Reverse	ATTTTATATTATTCATACAATCA

[a]The oligonucleotides for viral RNAs detection were designed according to reference genomes of TMV (#NC_001367), CGMMV(#D12505), ORSV (#X82130), CMV RNA1 (#D00356), TRV RNA1 (#AF166084), TCV (#M22445), TNV (#AY546104) and PVX (#EF423572).

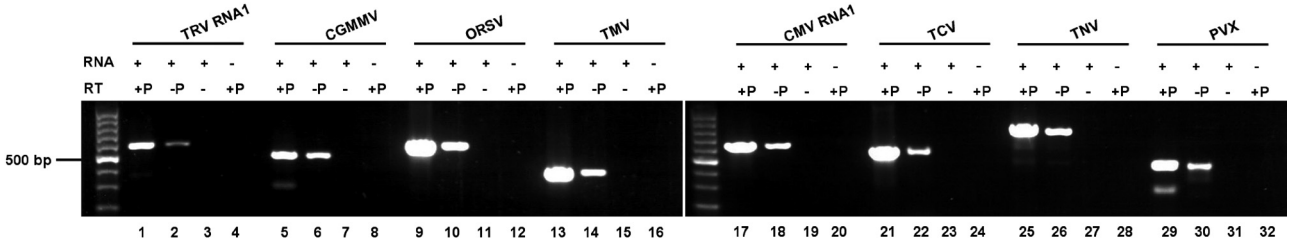

Fig. 1
Endogenous priming occurs during reverse transcription of the viral RNAs from eight different plant RNA viruses
One microgram of total RNA extracted from the virus-infected leaves of *N. benthamiana* by using Trizol was converted into cDNA in the presence or absence of specific RT primer (RT, +/-P). As a control, cDNA synthesis was carried out without addition of RT enzyme (RT -) or template RNA (RNA -). Following the RT step, the standard PCR (30 cycles) was performed and the resulting amplification products along with the 100 bp ladder were electrophoresed on a 1.2% agarose gel.

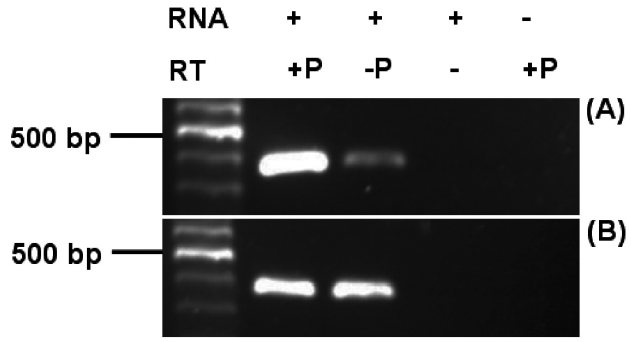

Fig. 2
Self-priming occurs during reverse transcription of the column purified RNA (panel A) as well as *in vitro*-transcribed RNA (panel B)
cDNA synthesis was performed on 1 μg of mini column purified total RNA from the virus-infected leaves of *N. benthamiana* or 100 ng *in vitro* TMV RNA with or without primer (RT, +/-P). Control experiments were carried out with no RT enzyme addition (RT -) or in the absence of template RNA (RNA -). After the RT step, the standard PCR (30 cycles) was performed and the resulting amplification products along with the 100 bp ladder were electrophoresed on a 1.2% agarose gel.

TMV-6395-76 (Table 1) and the following PCR program: 3 min at 94°C, followed by 30 cycles of 94°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec, and then 72°C for 10min. The resulting amplicons were electrophoresed on a 1.2% agarose gel stained with ethidium bromide and visualized under UV light. As expected, cDNA synthesis upon the use of TMV-6395-76 as an RT primer allowed the identification of a PCR product corresponding to nts 6023-6395 of TMV gRNA (Fig. 1, lane 13). Interestingly, cDNA synthesis in the absence of the RT primer generated a same size PCR product (Fig. 1, lane 14), and DNA sequencing revealed that the product indeed corresponded to the 3' termini of TMV gRNA. To exclude the possible DNA contamination or RT activity of *Taq* polymerase, we conducted two additional control experiments, in which RT reaction was performed in the absence of RT enzyme or template RNA. As expected, no visible PCR product was detected (Fig. 1, lanes 15 and 16). Using the same strategy, identical results were obtained after RT-PCR analysis of seven other plant RNA viruses (Fig. 1, lanes 1–12 and 17–32), including two additional tobamoviruses,

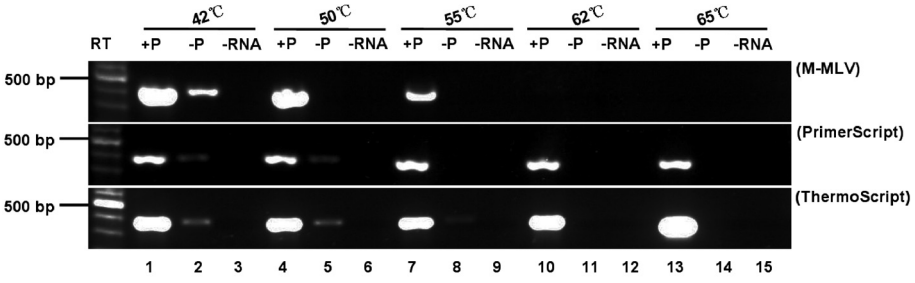

Fig. 3
The effect of the temperature on reverse transcriptase-mediated cDNA synthesis
Three different commercial reverse transcriptases, M-MLV (Promega), PrimeScript (Takara) and ThermoScript (Invitrogen) were included. Using 1 μg of total RNA prepared from the TMV-infected *N. benthamiana* leaves as template, RT reactions were individually preformed at 42°C, 50°C, 56°C, 62°C, and 65°C with or without specific RT primer (RT, +/-P). As a control, cDNA synthesis was carried out in the absence of the template RNA (-RNA). Following the RT step, the standard PCR (30 cycles) was performed and the resulting amplification products were electrophoresed on a 1.2% agarose gel along with the 100 bp ladder.

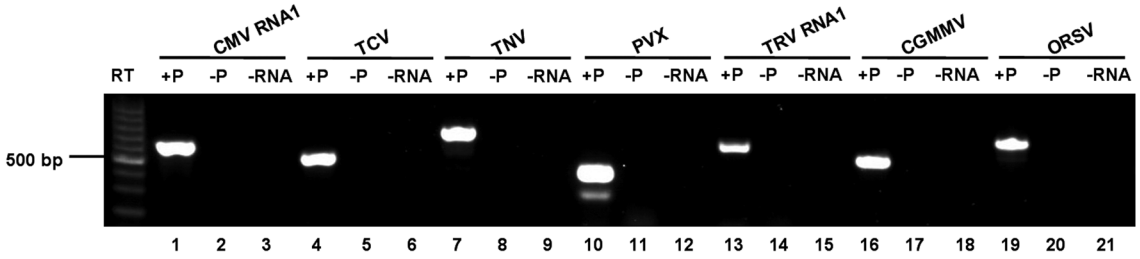

Fig. 4
Effective inhibition of RNA self-priming in a broad range of plant viral RNAs by using ThermoScript reverse transcriptase to reverse-transcribe RNA at high temperature

One microgram of total RNA extracted from the virus-infected *N. benthamiana* leaves was used as the template, and RT reactions were preformed at 62 °C with or without specific RT primer (+/-P). As a control, cDNA synthesis was carried out in the absence of the template RNA (-RNA). Following the RT step, the standard PCR (30 cycles) was performed and the resulting amplification products along with the 100 bp ladder were electrophoresed on a 1.2% agarose gel.

odontoglossum ring-spot virus (ORSV) and cucumber green mottle mosaic virus (CGMMV), as well as cucumber mosaic virus (CMV; the genus *Cucumovirus*), tobacco rattle virus (TRV; the genus *Tobravirus*), turnip crinkle virus (TCV; the genus *Carmovirus*), tobacco necrosis virus (TNV; the genus *Necrovirus*) and potato virus X (PVX; the genus *Potexvirus*). The primers used are listed in Table 1. Taken together, these data conclusively demonstrate that the primer-independent cDNA synthesis is a basic phenomenon in RT-PCR detection of many plant RNA viruses, and probably occurs with endogenous priming at the 3' termini of viral RNA.

As far as is known, the endogenous priming was likely caused by the intrinsic property of the template RNA or cellular small nucleic acids associated with reverse transcriptase and template RNA preparations. Regarding the fact that M-MLV reverse transcriptase used in the current study contains only few small RNA contaminations (Agranovsky, 1992; Moison *et al*., 2011), the possibility that reverse transcriptase serves as a source of non-specific cDNA synthesis could be excluded herein. Accordingly, the following experiments were performed to clarify whether cellular small nucleic acids within RNA preparations contributed to the primer-independent cDNA synthesis. Taking the advantage of RNeasy Plant Mini Kit (QIAGEN) in removal of small nucleic acids, we first prepared the template RNA from the TMV-infected *N. benthamiana* leaves by utilizing this kit instead of Trizol used above. As a result, this strategy failed to avoid the primer-independent cDNA synthesis (Fig. 2a). Additionally, to further rule out the influence of trace amounts of cellular nucleic acids, we prepared *in vitro* RNA transcripts of TMV U1 from linearized plasmids as described (Dawson *et al*., 1986). PCR amplification was first performed by using 100 ng of the DNaseI-treated RNA transcripts as a template without prior reverse transcription, ensuring the complete removal of the DNA template (data not shown). However, the primer-independent event remained in the RT-PCR analysis of the pure RNA transcripts (Fig. 2b). Taking all the observations into account, we determined that small nucleic acids are poorly associated with the endogenous priming, whereas the RNA self-priming should account for the primer-independent cDNA synthesis.

Previous reports showed that the increase of RT reaction temperature is an effective approach to attenuate or eliminate the RNA self-priming in cDNA synthesis (Haddad *et al*., 2007; Moison *et al*., 2011). We therefore adopted this strategy to overcome the RNA self-priming that occurs in RT-PCR detection of plant RNA viruses. Meanwhile, given that increasing the temperature would inevitably affect the efficiency of RT, in addition of M-MLV used above, PrimeScript (Takara) and ThermoScript (Invitrogen), two additional commercial reverse transcriptases with higher thermal stability (the recommended reaction temperature for M-MLV is of 42°C, whereas 50 °C for PrimeScript and 62°C for ThermoScript) were included herein to systematically compare their properties in RT reaction. Using total RNA (1 μg) of the TMV-infected *Nicotiana benthamiana* leaves as a template, RT reactions were preformed at progressively increased temperature of 42 °C, 50 °C, 56 °C, 62 °C, and 65 °C, respectively. The subsequent PCR amplification indicated that all reverse transcriptases compromised to the RNA self-priming in cDNA synthesis at lower reaction temperatures, which, however, could be successfully eliminated or reduced to below detection level by increasing RT reaction temperature (Fig. 3). Notably, with increasing RT temperature, the activity of M-MLV was significantly attenuated, and no PCR product was amplified at all once the RT temperature reached 62°C (Fig. 3, lane 10). In contrast, both PrimeScript and ThermoScript remained active even at 65°C (Fig. 3, lane 13). Particularly, ThermoScript, as a thermosTable enzyme, seemed to become more efficient when the RT temperature was increased from 42°C to 65°C. Hence, ThermoScript was applied in RT-PCR detection of the other seven plant

RNA viruses mentioned above. When cDNA synthesis was performed at 62°C but in the absence of the RT primer, the subsequent PCR amplification identified no visible product (Fig. 4, lanes 2, 5, 8, 11, 14, 17, and 20), indicating that increasing RT temperature is useful in preventing self-priming on a broad range of plant viral RNAs.

In conclusion, we show here that the primer-independent cDNA synthesis occurs widely during RT-PCR detection of plant RNA viruses, and is most likely caused by RNA self-priming. In view of the identification or diagnosis of plant RNA viruses, RNA self-priming is unlikely to affect the final result of the assay, and could even be beneficial, since it can amplify the signal and thus increase the sensitivity of the assay. However, once the aim of RT-PCR is to test the specific strand of the sense-antisense viral RNA pairs or some specific viral RNA molecules such as the TMV RNA with poly(A) tails, this event may give inaccurate or false positive results, leading to misinterpretation. While increasing RT temperature could eliminate or reduce RNA self-priming, setting up the appropriate negative controls remains essential to ensure the specificity of the RT-PCR reaction.

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