MULTIPLEX RT-PCR REACTION FOR SIMULTANEOUS DETECTION OF *TOMATO TORRADO VIRUS* AND *PEPINO MOSAIC VIRUS* CO-INFECTING *SOLANUM LYCOPERSICUM*

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Abstract: The tomato (*Solanum lycopersicum* L.) is cultivated all over the world and is a vegetable of significant economic importance. However, an increased production of the vegetable is directly connected with an elevated occurrence of pathogens limiting the production efficiency of the vegetable. Both, *Tomato torrado virus* and *Pepino mosaic virus* have been found to be serious disease factors. When not controlled, these viruses can significantly decrease tomato cultivation. In this article, we report a multiplex reverse transcription-polymerase chain reaction (RT-PCR) protocol for simultaneous detection of both, *Tomato torrado virus* (ToTV) and *Pepino mosaic virus* (PepMV) in virus infected plants. The assay was designed to specifically amplify the conserved regions of genomic ribonucleic acid (RNA) of both viruses. Moreover, the glycerandehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control of amplification to exclude false-negative assay results. High-resolution melt analysis of generated RT-PCR products was additionally performed to increase sensitivity and double-check the specificity of the reaction without the need of subsequent complementary deoxyribonucleic acid (cDNA) sequencing.

Key words: high-resolution melt (HRM), *Pepino mosaic virus*, RT-PCR, *Tomato torrado virus*

INTRODUCTION

Tomato torrado virus (ToTV) is a serious pathogen of *Solanum lycopersicum* L. To date, several isolates of the virus have been identified in Europe, the United States, and Australia (Gambley *et al.* 2010) as well as in Poland (Pospieszny *et al.* 2007; Budziszewska *et al.* 2008). The *Tomato torrado virus* belongs to the genus *Torradovirus*, in the family Secoviridae. Torrado viruses have spherical virions and two genomic ribonucleic acid (RNA): RNAs: RNA1 and RNA2, that are ca. 7.8 kb and 5.4 kb nucleotides in length. In the presence of *Bemisia tabaci* (Amari *et al.* 2008) or *Trialeurodes vaporariorum*, ToTV efficiently spreads (Pospieszny *et al.* 2010 and personal communication). It was also reported, that ToTV can be introduced to its host by mechanical inoculation (Amari *et al.* 2008).

Symptoms of ToTV infection in tomato begin with a yellowing of the leaflet bases that turn into regular necrosis of the whole leaves and fruits. The economic value of tomatoes then dramatically decreased (Pospieszny *et al.* 2010). *Tomato torrado virus* is considered to be a serious disease agent of tomatoes. Any emerging of the virus should be constantly monitored. Importantly, *Tomato torrado virus* was also found in co-infection with *Cucumber mosaic virus* (CMV), (Herrera-Vasquez *et al.* 2009), *Potato virus Y* (PVY), *Tomato spotted wilt virus* (TSWV), *Tomato mosaic virus* (ToMV), *Parietaria mottle virus* (PMoV), *Tomato chloro-sis virus* (ToCV) and *Tomato yellow leaf curl virus* (TYLCV, Alfaro-Fernández *et al.* 2010a) *Pepino mosaic virus* (PepMV), and other serious agents of viral disease of tomato (Gomez *et al.* 2010). *Pepino mosaic virus* belongs to the *Potexvirus* genus and has single-stranded RNA encapsided into filamentous particles (Hanssen and Thomma 2010). Comparative analysis of known, complete, genome sequences of PepMV revealed their high variability and allowed for four genotypes of the virus to be distinguished. The four genotypes are: European (EU), Chilean (Ch2), Peruvian (LP), and American (US), sharing nucleotide sequence identities ranging from 78 to 95% (Hanssen and Thomma 2010).

Tomatoes infected with *Pepino mosaic virus* show diverse symptoms depending on the virus isolate invading the host plant (Hanssen *et al.* 2009). The most severe isolates of PepMV cause fruit marbling, discoloration or even opening, and lead to steam chlorosis and necrosis (Hanssen *et al.* 2009; Hasiow-Jaroszewska *et al.* 2009). Moreover, PepMV is efficiently transmitted mechanically, which greatly improves the chances of this virus spreading in both field and greenhouse tomato cultivations. Despite of the great progress that has been made in understanding the molecular biology of plant virus-host interactions, there is still no efficient method to eliminate virus particles already existing in infected tissues. Controlling the occurrence of the virus in places where plant

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cultivation is highly developed, is mostly based on prevention and identification of the sources of the viruses in seedlings to be planted as well as eradication of infected plants, and control of the virus vectors.

There are a number of assays for the identification of plant viruses in infected tissues. The techniques can be divided into two basic types: immunoassays, for instance Enzyme-Linked Immunosorbent Assay (ELISA), or nucleic acid based protocols: Southern or northern hybridization, polymerase chain reaction (PCR) or reverse transcription-polymerase chain reaction (RT-PCR). Among them, the PCR-based techniques show high specificity and sensitivity. The time which takes place between the sample preparation and obtaining of the results is relatively short. In laboratories involved in pathogens detection of both plants and animals, the PCR method is currently being commonly used and recommended. Moreover, one of advantages of PCR is its ability to multiplex, which means that during a single thermal reaction several targets can be detected and analyzed, and this cannot be achieved by ELISA-based methods.

In our study, we suggested a multiplex RT-PCR protocol for simultaneous detection of ToTV and PepMV existing in co-infection in plant samples, particularly, in *S. lycopersicum* – a natural host of the pathogens. Detection of the internal control of *S. lycopersicum* the glycerandehyde 3-phosphate dehydrogenase (GAPDH) mRNA sequence, performed together with target viral RNAs, allows the false-negative results to be eliminated, and correctly evaluates the obtained results. Moreover, the conventional RT-PCR stage of the assay is followed by high-resolution melt (HRM) analysis of reaction products. This additional modification is advantageous, time-saving, and enables the reaction specificity to be confirmed without the labor-consuming step of cDNA sequencing.

MATERIALS AND METHODS

In the study, two Polish (Wal03 and Kra), (Budziszewska *et al.* 2008) and four Spanish (ALM-04, MUR-05, GNC-06, TEN-07), (Alfaro-Fernández *et al.* 2010b) isolates of ToTVs maintained on different hosts, and two isolates of PepMV (P11 and P22) maintained on *Solanum lycopersicum* plants (Grace and Betalux varieties susceptible to ToTV and PepMV), were used.

The solution TriReagent (Life Technologies) was used according to the manufacturer's recommendation to isolate total RNA from plants. Briefly, leaf sample (of up to 100 mg) was homogenized. A mixture of TriReagent and chlorophorm was used to extract the RNA. Isopropanol was used to precipitate RNA from the aqueous layer. The obtained pellet was resuspended in RNase-free water. Subsequently, ca. 3 µg of the RNA was subjected to denatured agarose gel to estimate the integrity of the RNA.

Two µg of the RNA was used for synthesis of cDNA, using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The sample of RNA was combined with 200 ng of random hexameres (Thermo Scientific). Next, 12 µl of the mixture was incubated at 65°C for 5 minutes to denature the RNA and primers. Afterwards, the mixture was rapidly cooled on ice and 4 µl of 5x Reaction Buffer, 2 µl of 10 mM dNTPs (Novazym), 20 U RiboLock RNase Inhibitor (Thermo Scientific), and 200 U of RevertAid M-MuLV Reverse Transcriptase (Thermo Scientific) were added to the mixture. Random primers were annealed to RNA at 25°C for 10 minutes and cDNA synthesis was conducted for 60 minutes at 42°C, followed by thermal inactivation of the enzyme.

Primers design

To specifically amplify partial sequence of ToTV and PepMV, the conserved genomic regions of the viral RNAs were selected for the design and annealing of the primers. Homology of the suggested primer sequences was analyzed using Basic Local Alignment Search Tool (BLAST) to avoid primer-template mismatches and to increase their specificity to the wide range of ToTV and PepMV isolates. The primers were designed to obtain relatively short PCR products that would distinctly differ in length after electrophoresis. Multiplexing of the reaction would then be possible. Finally, the target gene sequences of the Vp26 coat protein (CP) subunit and triple gene block protein (TGB) of ToTV and PepMV, respectively, were chosen for RT-PCR (Table 1).

Additionally, another primer pair was designed to amplify the fragment of the sequence of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control of PCR amplification (Wieczorek *et al.* 2013).

Polymerase chain reaction

Specific amplification of target cDNAs of ToTV, PepMV, and GAPDH was conducted by polymerase chain reaction. Polymerase chain reaction was performed in a final volume of 15 µl and reaction mixture consisted of 1× Allegro *Taq* Polymerase Buffer (Novazym) containing 70 mM Tris-HCl pH 8.6, 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, forward and reverse primers mixture (Table 1), 0.5 mM deoxyribonucleotides, 1 U of Allegro *Taq* DNA Polymerase (Novazym), and 1 µl (equivalent of ca. 100 ng of RNA) of cDNA. After initial denaturation (3 min) at 95°C, 35 cycles of the three-step reaction were performed as follows: 30 s at 95°C, 30 sec annealing of primers (gradient temperature 51–72°C), and 30 s at 72°C. Gradient RT-PCR revealed that optimal temperature during the annealing step should be set at 72°C. For this reason, all subsequent RT-PCR assays were conducted using a two-step reaction: 30 s 95°C and 30 s 72°C cycled 35 times. After the last cycle, a final extension at 72°C for 10 min was performed. The PCR mixture was loaded into 1% agarose gel supplemented with Midori Green dye (NIPPON Genetics). The mixture was then electrophoresed in the TBE buffer. DNA of estimated size was extracted from the gel (Wizard® SV Gel and PCR Clean-Up System, Promega) and ligated with pGEM-T Easy vector (Promega). The ligation mixture was used to transform competent *E. coli* TOP10 cells (Invitrogen). After colony PCR, performed with the previously mentioned primers, plasmids were isolated from *Escherichia coli* transformants (Macherey-Nagel). The presence of DNA inserts was evaluated again after plasmid digestion, using *EcoRI* enzyme (Thermo Scientific). Plasmid DNA was sequenced (DNA Research Center, Poznań, Poland) in both reverse and forward directions.

using M13rev or M13for sequencing primers, respectively. The obtained cDNA sequence was analyzed using BLAST to verify the origin of the sequences.

Real-time HRM-PCR

The real-time PCR reaction was followed by high resolution melt analysis (HRM) of products. The reaction mixture consisted of the same reagents as mentioned previously in the PCR protocol, however, the mixture was additionally supplemented with DNA intercalating dye – EvaGreen (1x, Biotium). The thermal profile of reaction was the same as for RT-PCR, except that the final elongation step was substituted by a gradual increase in temperature (0.1°C/s) that allowed the quality of the PCR products on the high resolution level to be analysed.

RESULTS

In the study we suggested a multiplex RT-PCR protocol for simultaneous detection of *Tomato torrado virus* and *Pepino mosaic virus* existing in co-infection on host plants – *S. lycopersicum*. To obtain our goal, we analysed nucleotide sequences of genomic RNAs of the viruses, and on basis of the performed comparative analysis, two pairs of oligonucleotide primers were designed (Table 1). Comparative global BLAST *in silico* analysis of the primers`sequences has indicated, that the oligonucleotides TOTA and TOTB are able to hybridize to all known complete genomic sequences (Polish and Spanish isolates) of ToTV deposited in Gene Bank. The same analysis, performed using the primers PepA and PepB, revealed their high similarity with known Polish and Spanish isolates of PepMV (data not shown). Furthermore, we performed

optimization of the suggested multiplex RT-PCR reaction to detect the two pathogens in *S. lycopersicum*. To do this, the total RNA was isolated from infected plants and reverse transcribed. The obtained cDNA was used in further analyses as a PCR template. Gel electrophoresis of gradient PCR products has shown that during the annealing step of RT-PCR performed at 72°C, no unspecific products or primers-dimers structures were detected (data not shown), and therefore the temperature was optimal. All subsequent RT-PCR reactions, including HRM-RT-PCR experiments, were performed at 72°C of annealing temperature. To verify specificity of optimized multiplex PCR, we conducted triplex RT-PCR reactions with three primer pairs and cDNAs synthesized on RNA templates isolated from *S. lycopersicum* infected with either ToTV or PepMV. As a negative control, we used cDNA of healthy plants, while the mixture of cDNAs of ToTV and PepMV were used as double template samples (ToTV/PepMV co-infection). As indicated, (Fig. 1) 217 bp DNA bands, referring to the internal control (GAPDH), were identified in all of the analysed samples, while additional bands of 366 bp and 270 bp were detected only in *S. lycopersicum* infected with ToTV or PepMV, respectively. Three DNA bands of estimated sizes were detected only in tomato samples co-infected with ToTV and PepMV. DNA sequencing of 217 bp, 270 bp, and 366 bp DNA fragments confirmed the presence of specific products for Vp26, TGB and *GAPDH* of ToTV, PepMV, and the internal control, respectively. Analysis of plant samples derived from Spain indicated single and double-infections (Fig. 2). This signifies that optimized RT-PCR was specific, and no unspecific products were identified within the analysed samples.

Table 1. Primers used in the multiplex RT-PCR. Low indexes indicate the position of primers within virus genome (according to the complete genome sequences of ToTV and PepMV deposited in Gene Bank numbers: EU563947.1 and HQ650560.2, respectively)

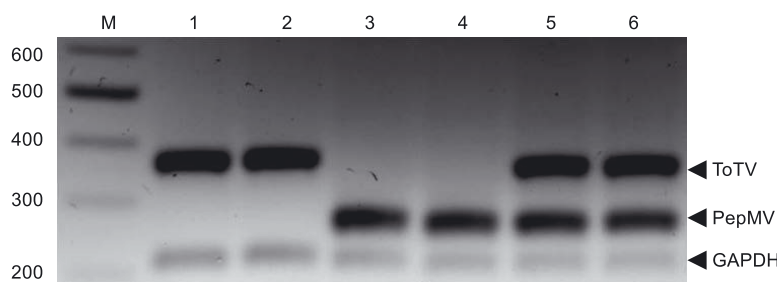| Primers`ID	5`→ 3` sequence	Final concentration in the RT-PCR mix [μM]	Estimated product length [bp]
ToA	$_{3098}$GACTGGCACTTGCGTGTTTGAG$_{3119}$	0.16	399
ToTB	$_{3463}$GTGCCCCCAAGGACCAAATCTT$_{3442}$		
PepA	$_{5114}$TGCCAGGTCTGACTCCTAGAGC$_{5135}$	0.5	270
PepB	$_{5383}$TGGTGAGGACAATACCCARTGT$_{5362}$		
GAPDH1	GCTGGTGCTGACTTCGTTGTTG	0.16	217
GAPDH2	TAGCCAAAGGTGCAAGGCAGTT		

Fig. 1. Triplex RT-PCR assay for identification of ToTV, PepMV and GAPDH in *S. lycopersicum*. M – DNA mass marker [base pairs], 1–2 ToTV single infection with Kra and Wal03 isolates, respectively, 3–4 PepMV single infection with P11 and P22 isolates, respectively, 5–6 double infection. Arrows indicate specific RT-PCR products
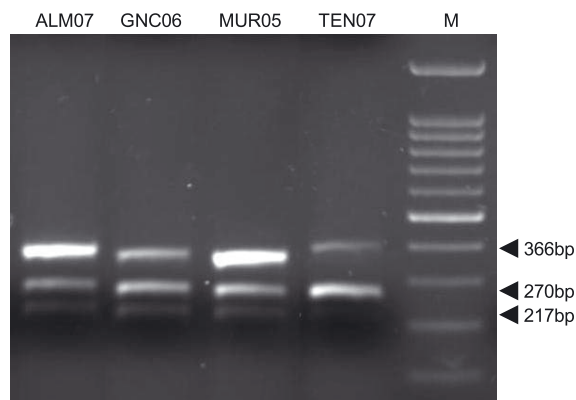
Fig. 2. Evaluation of the efficiency and specificity of the triplex RT-PCR for identification of ToTV and PepMV protocol on the plant samples derived from Spain. Plant samples were tested for the presence ToTV and PepMV frequently found in co-infection. M- DNA mass marker, ALM04, GNC06, MUR05 and TEN07- Spanish plant samples infected with viruses
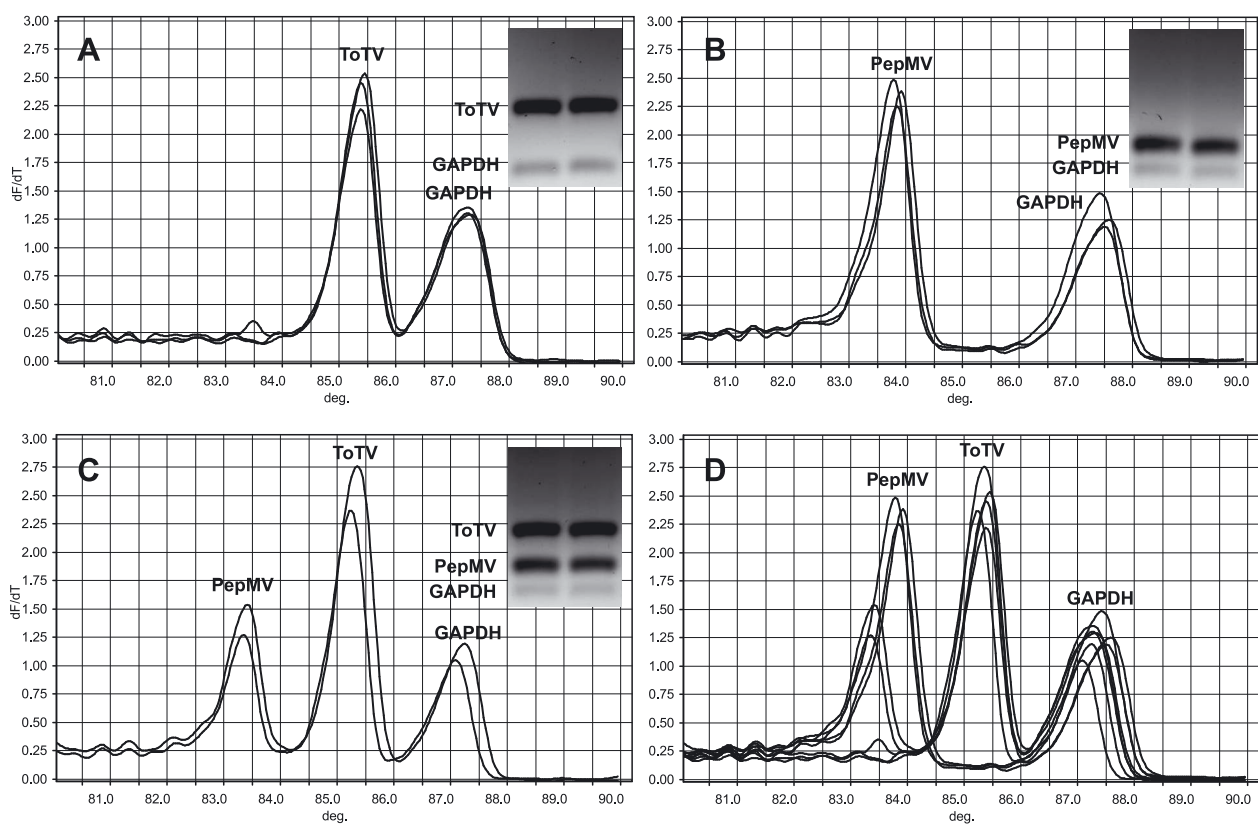

Fig. 3. HRM analysis of triplex RT-PCR. Characteristic melting peeks of ca. 83.63°C, 85.25°C and 87.25°C are associated with presence of ToTV, PepMV and GAPDH in the samples, respectively. Double peek represents single ToTV (panel A) or PepMV (panel B) infection. Triple peek represents co-infection (panels C and D)

In addition to the standard RT-PCR reaction described above, the reaction can be performed using HRM real-time PCR protocol. It can serve to improve verification of the presence of specific products of RT-PCR. Therefore, we performed high resolution melt analysis of amplified cDNAs. We expected, that due to differences in the length of the RT-PCR products and their nucleotide composition, three main melting peaks, visibly distinguishable, should be identified. Indeed, melting points of specific PCR products were generated at ca. 83.63°C, 85.25°C and 87.25°C of ToTV, PepMV and *GAPDH*, respectively. In samples infected only with one virus, two peaks were detected, whereas three peaks were identified in samples co-infected with PepMV and ToTV (Fig. 3).

DISCUSSION AND CONCLUSIONS

Tomato is a crop of high economic importance. It is important to constantly monitor the occurrence of pathogens infecting the plants, since pathogens lead to a decrease in crop production. The appearance of symptoms representing unfavorable phenotypes, for instance due to virus infection, diminish the tomato's consumer values. *Tomato torrado virus* is a relatively recently characterized

virus, and its biology is rather poorly understood. However, since 2007, ToTV has been considered to be a dangerous disease agent (Verbeek *et al*. 2007). Moreover, ToTV can efficiently infect tomatoes also in co-infections with another tomato virus – PepMV (Gomez *et al*. 2010). To date, plant protection against viral diseases was based on using plant varieties resistant to viral pathogens, or on prevention that was based mostly on the control of the occurrence of virus vectors, the identification of virus infected seedlings or on limiting the possibility of transmitting the virus through the use of contaminated hands or tools. In this study, we recommended a new protocol, based on multiplex RT-PCR, for simultaneous detection of ToTV and PepMV infections in plants. In comparison to the conventional singleplex RT-PCR reaction - based on identification of the viruses separately (one virus per tube), the multiplex RT-PCR assay has proved a time- and labor-saving method. Diagnostics are then possible of more than one virus in one reaction tube, that can be used in a wider range of applications (Yin *et al*. 2012).

As we have shown, three pairs of primers efficiently amplified targets: the partial sequence of Vp26, TGB, and *GAPDH* (Fig. 1) of ToTV, PepMV, and *S. lycopersicum*, respectively. Regarding the TOTA/TOTB primers, we suspected them of having versatile hybridizing properties to ToTV cDNA, due to the high similarity of known nucleotide sequences of *Tomato torrado viruses* (Budziszewska *et al*. 2008). Moreover, as described in the study, primers PepA and PepB efficiently amplified P11 and P22 isolates of PepMV belonging to CH2 and EU genotypes, respectively. The described primers hybridize to the target even if the 1–2 nucleotide internal mismatches are present within the oligonucleotide-template duplex. Considering the fact that specificity of PCR reaction is directly related to primer-template stability, particularly with its 3' end from which DNA polymerization starts (Bru *et al*. 2008; Stadhouders *et al*. 2010), we concluded, that the described primers PepA/B would be specific to all other PepMV genotypes. This is especially true in the case of pathotype US2 where there is 100% identity between designed primers and the target sequence in the viral genome.

The obtained RT-PCR products were clearly distinguishable after agarose gel electrophoresis, when Polish isolates of the viruses had been taken for analysis. The ToTV and PepMV were also identified in single or double infection in plant material derived from Spain (Fig. 2). The use of the internal control during RT-PCR is important, especially when the assay has to confirm or exclude the presence of an analyzed target in a sample (Menzel *et al*. 2002; Chen *et al*. 2011). The use of the internal control minimizes the risk of obtaining false-positive results that might be misinterpreted. Multiplexed detection protocols, that save experiment performance time, and reagent costs, are widely utilised in real-time RT-PCR format. In the assays, differently labeled molecular probes, specific for the target to be detected, are used. This, however, is directly related to the increase in the overall costs of the assay, and requires additional primers that might influence the thermodynamics of a reaction and its optimization. We showed that the assay we described can be used in conventional RT-PCR as well as in HRM RT-PCR for-

mat (Figs. 1–3). The real-time RT-PCR modification of the assay can be advantageous especially when pathogen is thought to occur on low titers in plants (Agindotan *et al*. 2007; Quito-Avila and Martin 2012). The use of a DNA classic intercalating dye, like SYBR green, restricts the number of targets to be detected in a single-tube assay. A solution is to use the next-generation DNA saturating intercalating dyes, for instance EvaGreen, that greatly improve the resolution of melt analysis of PCR products (Mao *et al*. 2007). The qualitative differences can then be distinguished between particular PCR amplicons originated from diverse targets or hosts (Gurtler *et al*. 2012; Cheng *et al*. 2013) without the subsequent use of agarose gel electrophoresis or DNA sequencing. In the suggested study, HRM analysis of amplified DNAs displayed distinct shifts in melting peaks specific for target samples (Fig. 3). The characteristic melting temperature is unique for each PCR product that differ in length and nucleotide composition, and this was also verified by *in silico* thermodynamics calculations of ToTV- PepMV- and GAPDH-derived RT-PCR products (Integrated DNA Technologies, Owczarzy *et al*. 1997). These noted factors confirmed the specificity and high sensitivity of the assay. Additionally, ToTV- and PepMV- derived cDNAs inserted within plasmids used in the assay, provide excellent reference sequences (Tajiri-Utagawa *et al*. 2009) to which normalized HRM melting curves of tested samples can be compared.

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