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Quantitative Determination of *Cucumber Mosaic Virus* Genome RNAs in Virions by Real-Time Reverse Transcription-Polymerase Chain Reaction

Jun-Li FENG[1], Shao-Ning CHEN[1], Xiang-Shan TANG[1], Xian-Feng DING[1], Zhi-You DU[2], and Ji-Shuang CHEN[1,2]*

[1] *Institute of Bioengineering, Zhejiang Sci-Tech University, Hangzhou 310018, China;*
[2] *College of Life Sciences, Zhejiang University, Hangzhou 310029, China*

Abstract A real-time RT-PCR procedure using the green fluorescent dye SYBR Green I was developed for determining the absolute and relative copies of *cucumber mosaic virus* (CMV) genomic RNAs contained in purified virions. Primers specific to each CMV ORF were designed and selected. Sequences were then amplified with length varying from 61 to 153 bp. Using dilution series of CMV genome RNAs prepared by *in vitro* transcription as the standard samples, a good linear correlation was observed between their threshold cycle (C_t) values and the logarithms of the initial template amounts. The copies of genomic RNA 1, RNA 2, RNA 3 and the subgenomic RNA 4 in CMV virions were quantified by this method, and the ratios were about 1.00:1.17:3.58:5.81. These results were confirmed by Lab-on-a-Chip and Northern blot hybridization assays. Our work is the first report concerning the relative amounts of different RNA fragments in CMV virions as a virus with tripartite genome.

Key words real-time reverse transcription-polymerase chain reaction; *cucumber mosaic virus*; genomic RNA; quantitative determination

Cucumber mosaic virus (CMV) is a species of the *Cucumovirus* genus within the family *Bromoviridae*. It has been long known that the virus has a genome structure consisting of three single-stranded messenger sense RNAs, (RNA 1, 2 and 3) and two subgenomic RNAs (RNA 4A and RNA 4). RNA 1 and RNA 2 encode components of viral RNA-dependent RNA polymerase (RdRp), the 1a and 2a proteins, with putative helicase and polymerase activities, respectively. RNA 3 encodes the 3a movement protein and the coat protein (CP), and the latter is expressed from RNA 4 as subgenomic RNA. In addition, the 2b protein is expressed from 3′-proximal sequences of RNA 2, via subgenomic RNA 4A. This protein is a pathogenicity-determinant and plays a role in the long-distance movement of CMV [1,2].

CMV has a very large host range, which is estimated to be over 1000 species in 85 families and has a worldwide distribution [3]. While the crop losses caused by CMV have increased greatly in past decades, its replication and pathogenicity mechanisms remain mostly undiscovered. Several publications have mentioned that CMV genomic RNAs do not accumulate in equimolar amounts in hosts, and that the relative ratios between them have a strong effect on the level of virulence and the level of virus accumulation [4–6]. Therefore, a rapid and sensitive method for reliably quantifying the amount of each CMV genomic RNA would aid the understanding of the mechanisms of viral replication and pathogenicity, as well as the interaction with hosts.

The amounts of CMV genomic RNAs were previously quantified by electrophoresis, Northern blot hybridization and RT-PCR [7]. However, these methods vary in their usefulness for accurate quantification, being either time consuming, laborious, insensitive, or having large results variations. Real-time RT-PCR is a new method developed in recent years. By adding the fluorescent signal to the reaction mixture, the whole PCR process is monitored through the increase of fluorescence and the absolute amount of target is calculated from a calibration curve. Due to its high sensitivity and reproducibility, real-time RT-PCR has been considered the most reliable method

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*Corresponding author: Tel/Fax, 86-571-86843196; E-mail, chenjs@zist.edu.cn

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currently available and is used widely in gene (e.g. transgene or pathogen) detection and quantification, and gene expression studies [8,9]. Its applications in virus researches are increasing, and many economically important plant viruses have been detected by this method [10–13]. But most of these studies are related simply to the qualitative detection of viruses, taking advantage of its broad quantification range (≥7 magnitudes) and low titration limitation. To date, there is no report on the quantification of different RNA fragments in multipartite viruses.

This paper describes the development of a real-time RT-PCR assay with SYBR Green I, which allows an accurate quantification of CMV genomic RNAs. Their copy number ratios in virions were calculated and the results were confirmed by Lab-on-a-Chip and Northern blot hybridization assays.

Materials and Methods

Plants and viruses

Nicotiana tobacum Huangmiaoyu tobacco plants were maintained under greenhouse conditions with a day length of 16 h at day/night temperature of 25 ºC/20 ºC. The young tobacco plants (four- to six-leaf stage) were used for virus inoculation by Fny-CMV (kindly donated by Peter PALUKAITIS, Scottish Crop Research Institute, Dundee, UK). Virions were purified from infected tobacco 17 d after inoculation [14]. Fny-CMV genomic RNAs were extracted from virions and from infected or healthy tobacco plants with the method as previously described [15]. The RNA samples were treated with DNase I and stored at −80 ºC.

Primer design and optimization

Two to four sets of primers were designed for each Fny-CMV ORF based on their sequences. Primers were first designed using Primer 5.0 software (PE Applied Biosystems, Foster City, USA) and then selected manually based on melting temperature (T_m), position and GC content in the last five bases. Virtual RT-PCR was run to screen for amplification efficiency and primer dimmer formation. The best primer set for each ORF was selected for subsequent experiments (**Table 1**).

Preparation of standard samples

To quantify Fny-CMV genomic RNAs, three sets of standard samples were transcribed from biologically active cDNA clones of Fny-CMV RNA 1 (pFny109), Fny-CMV RNA 2 (pFny209), and Fny-CMV RNA 3 (pFny309) *in vitro* with T_7 polymerase (Promega, Madison, USA), respectively. Standard samples of RNA 1 were used with 1a primer pair 1a-F/R, RNA 2 with 2a and 2b primer pairs 2a-F/R and 2b-F/R, RNA 3 with 3a and CP primer pairs 3a-F/R and CP-F/R in quantitative real-time RT-PCR. Transcription products were treated with DNase I firstly, and then RT negative PCR products were analyzed in triplicates to confirm there was no fluorescence resulting from either DNA template residue or RT step. Finally they were quantified to 400 μg/ml and stored at −80 ºC. The copy numbers could be determined by **Equation 1**.

$$N=C/(K\times330\times1.6601\times10^{-18}) \quad (1)$$

Table 1 Primers used for quantification of Fny-CMV ORFs by real-time reverse transcript-polymerase chain reaction

ORF	Primer sequence (5′→3′)	Range (position)
RNA 1		
1a ORF	1a-F, GGAGAGGAATGGGACGTGATATC	72 bp (1598–1669)
	1a-R, CAAAACTTTCCCATCGGTAACAG	
RNA 2		
2a ORF	2a-F, ATGAGCTCCTTGTCGCTTTTG	76 bp (361–436)
	2a-R, TTATTAAACGCAGGGCACCAT	
2b ORF	2b-F, CGGCGGAAGACCATGATTT	61 bp (2681–2741)
	2b-R, CCTTCCGCCCATTCGTTAC	
RNA 3		
3a ORF	3a-F, CTGATCTGGGCGACAAGGA	153 bp (454–606)
	3a-R, CGATAACGACAGCAAAACAC	
CP ORF	CP-F, CGTTGCCGCTATCTCTGCTAT	70 bp (1661–1730)
	CP-R, GGATGCTGCATACTGACAAACC	

where N was the copy number per μl, C was the concentration of sample (μg/μl), K was the length of target gene (nucleotide), 1.6601×10^{-18} was the transfer constant between Dalton and μg.

SYBR Green I reverse transcription-polymerase chain reaction

For RT-PCR, cDNA was synthesized in 10 μl reaction buffer containing 2 μl 5×M-MLV buffer, 0.5 μl specific primer (2 μM), 0.25 μl M-MLV RTase (200 U/μl; TaKaRa, Takara, Japan), 0.25 μl RNase inhibitor (40 U/μl; TaKaRa) and 1 μl template RNA. The thermal profile for RT was 42 ºC for 15 min and 95 ºC for 2 min.

The PCR was carried out in a 96-well plate in a reaction volume of 25 μl containing 12.5 μl 2×Premix *EX Taq* buffer (TaKaRa), 0.5 or 1 μl 50×SYBR Green I nucleic acid fluorescent dye (TaKaRa), 2 μl template cDNA, and forward and reverse primers at final concentrations varying from 0.1 μM to 1 μM. The thermal profile for PCR was 95 ºC for 10 s, followed by 40 cycles of 95 ºC for 10 s and 60 ºC for 30 s. Immediately after the final PCR cycle, a melting curve analysis was carried out to determine the specificity of the reaction by incubating the reaction mixture at 95 ºC for 15 s, annealing at 60 ºC for 20 s, and then slowly increasing the temperature to 95 ºC over 20 min [16]. The C_t used in the real-time PCR quantification was defined as the PCR cycle number that crossed an arbitrarily chosen signal threshold in the log phase of the amplification curve.

Standard curve for each ORF was generated using dilution series of standard samples as the RT-PCR template. The RNA extracted from virions was diluted 100 times and amplified along with standard samples under optimal concentrations, and the copy number of each ORF was calculated from standard curves according to its C_t value. Each sample had three replicates and all reactions were replicated three times independently to ensure the reproducibility of the results. The RNA extracted from healthy tobacco plants was used as a negative control. After the PCR, data were viewed and analyzed using the ABI 7000 Sequence Detection Software (PE Applied Biosystems). For each sample, the amplification plot and the corresponding dissociation curve were examined. Due to the variation in length and nucleotide composition, each amplicon had a unique T_m value [17].

Lab-on-a-Chip assay

Lab-on-a-Chip assay was carried out in Aglient 2100 Bioanalyzer System (Agilent Technologies, Palo Alto, USA), following the manufacturer's instructions of RNA 6000 Nano Reagents and Supplies (Agilent Technologies). The concentrations of Fny-CMV genomic RNAs were analyzed using 2100 Expert Software (Agilent Technologies) and the relative copy number ratios of them were calculated based on the molecular weight.

Northern blot hybridization

The standard samples and viral RNAs were denatured with formamide/formaldehyde prior to electrophoresis on a 1.6% agarose gel. Denaturation, electrophoresis, blotting to nitrocellulose membranes, hybridization, washing of the blot and autoradiography were carried out by standard procedures [18]. Probes were synthesized by Random primer label kit (TaKaRa). The RNA extracted from infected and healthy tobacco plants were used as positive and negative controls, respectively. The hybridization was replicated three times.

Results

Optimization of real-time RT-PCR

The real-time RT-PCR was first optimized by varying the concentrations of primers and SYBR Green I dye. It was found that 0.5 μl of SYBR Green dye was optimal for the reaction. Increasing the SYBR Green I dye concentration in the reaction mixture resulted in an error reading of the fluorescent signal from the amplified DNA. Also, 0.2 μM each of forward and reverse primers was the optimum concentration, as this gave the highest reporter fluorescence and the lowest C_t value.

Specificity of real-time RT-PCR

Real-time RT-PCR amplification of Fny-CMV ORFs with each primer set produced the expected amplicon. The predicted RT-PCR product was confirmed by agarose gel electrophoresis (data not shown). Dissociation curve analysis also demonstrated that each of the primer pairs tested amplified a single PCR product with a distinct T_m value.

Quantification of Fny-CMV genomic RNAs by real-time RT-PCR

Each ORF was amplified clearly and reproducibly by real-time RT-PCR (**Fig. 1**). The assay was proved to be highly reproducible, as demonstrated by low C_t standard deviation values between triplicates and a high correlation coefficient of the standard curves (R^2>0.99) (**Fig. 2**). Based on respective C_t values, the copy number of each

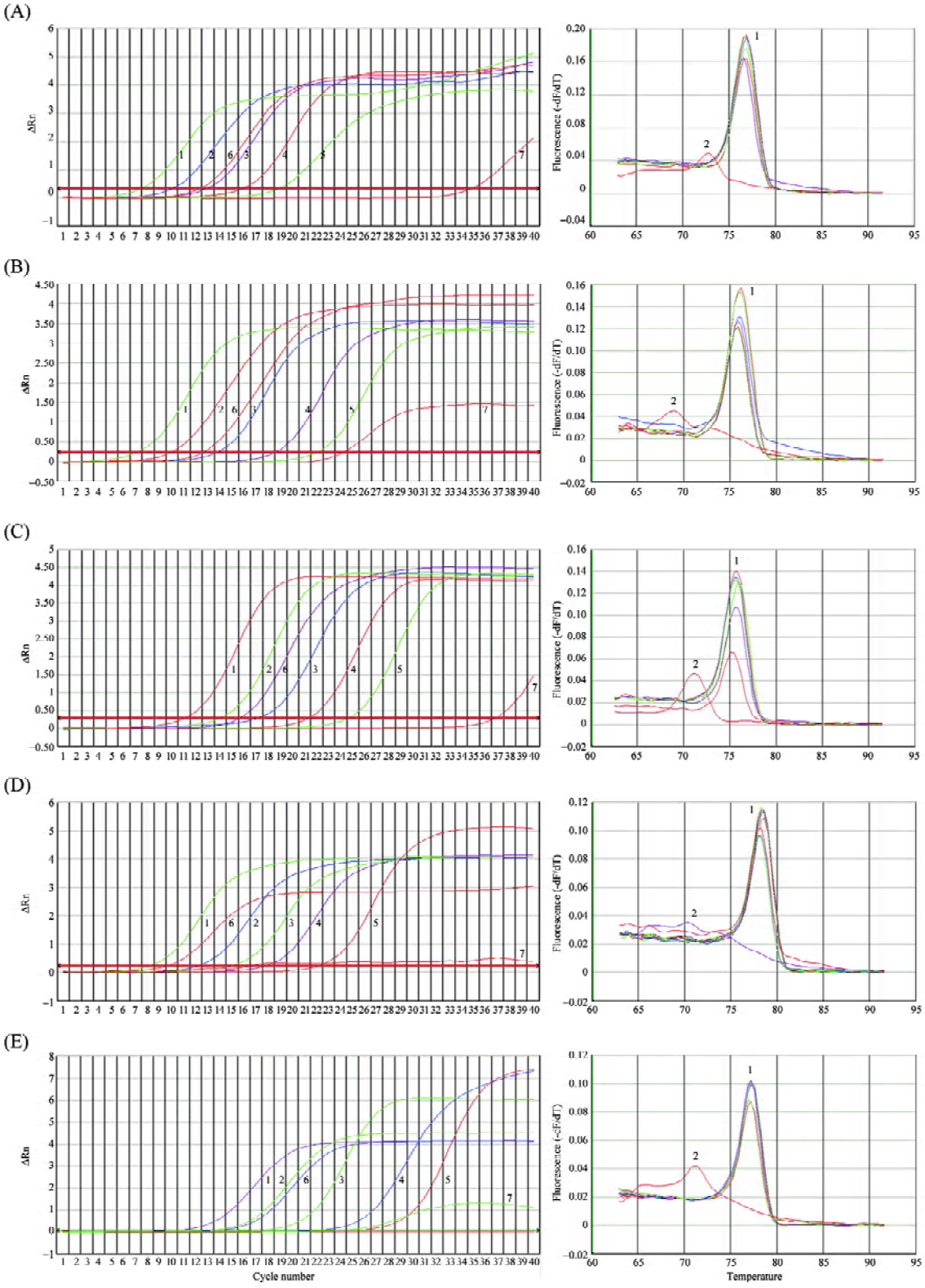

Fig. 1 Real-time RT-PCR cycle number amplification and dissociation curves of Fny-CMV ORFs

(A–E) 1a, 2a, 2b, 3a and CP ORFs respectively. Left, representative optic graph for the number of cycles versus the number of fluorescence units for each sample used to calculate the C_t value; 1–5, 10-fold serially diluted standard samples (1a ORF starting from 2.17×10^9 copies/μl, 2a ORF and 2b ORF starting from 2.39×10^9 copies/μl, 3a ORF starting from 3.29×10^8 copies/μl, CP ORF starting from 3.29×10^9 copies/μl); 6, RNA extracted from virions; 7, PCR-negative control. Right, corresponding dissociation curve analysis, with the results represented by a graph of number of fluorescent units versus temperature; the T_m value of each amplicon (1) and PCR-negative control (2).

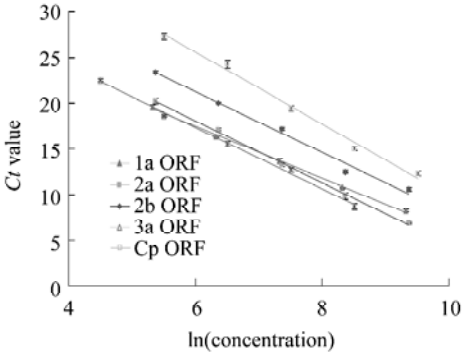

Fig. 2 Standard curves for the copy number of standard samples versus C_t values (n=3)

Standard samples of each ORF were serially diluted at one log unit intervals, corresponding to 1–5 in **Fig. 1** (left), the average C_t was plotted against the logarithm of the RNA concentration input into reverse transcription reaction. Regression equations were: 1a ORF, Y=–2.825X+34.424; 2a ORF, Y=–3.396X +38.455; 2b OFR, Y=–3.31X+41.107; 3a ORF, Y=–3.353X+37.416; CP ORF, Y=–3.93X+49.153.

Fny-CMV ORF was calculated with intra-group coefficient variations (CVs) of 0.76%–6.24% and inter-group CVs of 2.51%–11.37% [19]. Copy numbers of Fny-CMV RNAs 1 and 3 in virions were represented by the amounts of 1a ORF and 3a ORF, and RNA 4 was obtained by subtracting the amounts of 3a ORF from those of CP ORF (**Table 2**). A difference between the copy number of 2a ORF and that 2b ORF was observed [$(4.87\pm0.33)\times10^7$ vs. $(5.57\pm0.25)\times10^7$, CV=9.57%], but it was in the range of inter-group CVs, indicating absence of RNA 4A in Fny-CMV virions. Therefore, the copy number of RNA 2 was represented by the average amounts of 2a ORF and 2b ORF, and the copy number ratios between Fny-CMV RNA 1, 2, 3 and 4 in virions were determined to be 1.00: (1.17±0.11):(3.58±0.20):(5.81±0.31).

Table 2 Quantification of Fny-CMV ORFs in virions by real-time RT-PCR

ORF	C_t [a]	Copies per μl [b]
RNA 1		
1a ORF	12.25±0.11	$(4.47\pm0.50)\times10^7$
RNA 2		
2a ORF	12.00±0.10	$(4.87\pm0.33)\times10^7$
2b ORF	13.00±0.06	$(5.57\pm0.25)\times10^7$
RNA 3		
3a ORF	9.61±0.09	$(1.60\pm0.09)\times10^8$
CP ORF	13.26±0.12	$(4.20\pm0.11)\times10^8$

[a] The values are presented as mean±SD for three independent experiments. [b] The average initial copy number of each Fny-CMV ORF per μl of total RNA extracted from CMV virions. The result was obtained by 100×copy number per μl of each ORF in 100 times diluted virus RNA, which was calculated from regression equation of standard curve directly. Standard deviations for each ORF were calculated from an average of three replicates.

Comparison of the quantification results between real-time RT-PCR, Lab-on-a-Chip and Northern blot hybridization assays

To confirm the quantification results of real-time RT-PCR, RNAs extracted from virions were also analyzed in parallel by Lab-on-a-Chip and Northern blot hybridization assays. The results of Lab-on-a-Chip were shown in **Fig. 3**. Based on the electropherogram, the relative concentrations of Fny-CMV RNA 1, 2, 3 and 4 were obtained, and the copy number ratios between them were deduced to be 1.00:(1.23±0.08):(3.68±0.15):(5.79±0.65).

The results of Northern blot hybridization are shown in **Fig. 4**. Due to different hybridization efficiencies, the RNA 1, RNA 2 and RNA 3 standard samples with equal concentration (**Fig. 4**, lane 4) yielded different hybridization intensity. Thus when the relative concentration of each virus RNA was calculated, its hybridization intensity was divided by that of corresponding standard sample to eliminate this difference, and the copy number ratios between Fny-CMV RNA 1, 2, 3 and 4 were determined to be 1.00: (1.20±0.10):(4.03±1.07):(6.19±2.51).

The copy number ratios of Fny-CMV genomic RNAs in virions determined by real-time RT-PCR, Lab-on-a-Chip and Northern blot hybridization are compared in **Table 3**, revealing the largest result variations of Northern blot hybridization.

Discussion

There are two general approaches for real-time PCR: the specific and non-specific fluorescent reporting chemistries. Both display similar levels of sensitivity [20]. The use of a specific probe-based assay such as TaqMan-PCR requires high complementarity for probe binding, which might result in a failure to detect a high sequence variability in the probe-binding region, while non-specific assays using intercalating dyes such as SYBR Green I were found to be more reliable, flexible, simple, and of lower cost for detecting nucleic acid targets characterized by sequence variability, especially for RNA viruses. SYBR Green I is a minor groove DNA binding dye with a high affinity for double-strand DNA (dsDNA) and exhibits fluorescence enhancement upon binding to dsDNA. The ac-

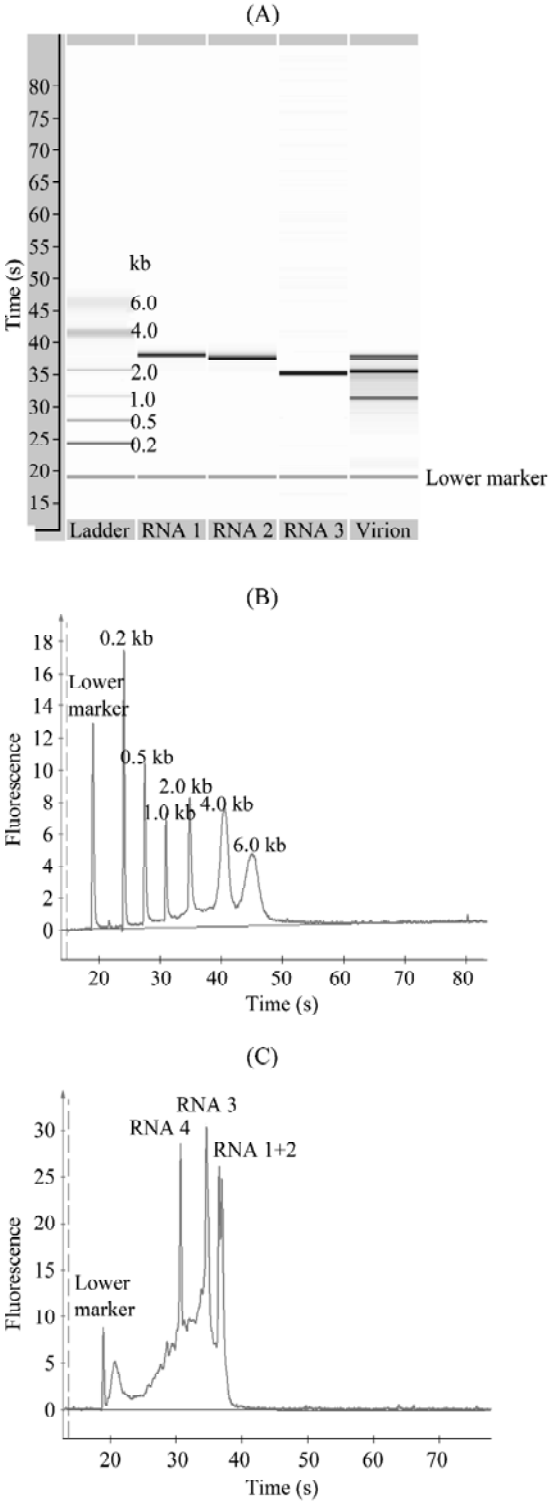

Fig. 3 Quantification of Fny-CMV RNAs by Lab-on-a-Chip assay

(A) Gel result. RNA 6000 Ladder (0.2, 0.5, 1.0, 2.0, 4.0, and 6.0 kb respectively) (Ambion, Austrin, USA) was used. (B) Corresponding electropherogram of RNA Ladder. (C) Corresponding electropherogram of Fny-CMV RNAs extracted from virions. Lower markers are internal standards and are used to align the ladder analysis with the individual sample analysis.

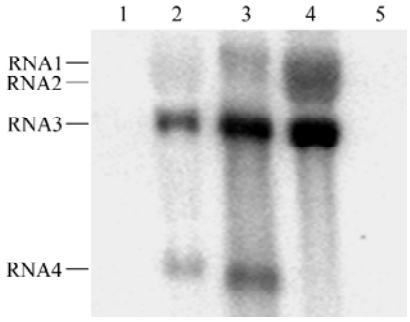

Fig. 4 Quantification of Fny-CMV RNAs by Northern blot hybridization

1, RNA extracted from healthy tobacco plants; 2, RNA extracted from infected tobacco plants; 3, RNA extracted from virions; 4, mixture of 200 ng of each standard sample; 5, negative control of *in vitro* transcription.

Table 3 The ratio of Fny-CMV genomic RNAs in virions determined by real-time RT-PCR, Lab-on-a-Chip and Northern blot hybridization

Sample	Copy number ratio		
	Real-time RT-PCR	Lab-on-a-Chip	Northern blot hybridization
RNA 1	1.00	1.00	1.00
RNA 2	1.17±0.11	1.23±0.08	1.20±0.10
RNA 3	3.58±0.20	3.68±0.15	4.03±1.07
RNA 4	5.81±0.31	5.79±0.65	6.19±2.51

cumulation of amplified DNA is measured by determining the increase in fluorescence over time, and this is followed by confirmation of results by melting curve analysis [21]. In this study, real-time RT-PCR with SYBR Green I was used for quantificating ORFs of Fny-CMV genomic RNAs, and the results showed that it is reliable in determining the copy number ratios between them.

The disadvantages of real-time RT-PCR (SYBR Green I) quantification was its indiscriminate binding to any dsDNA, which could result in fluorescence readings in the primer dimers and non-specific amplification, so the dependability of the assays relied greatly on the specificity of the amplification [22]. Therefore in this study, primers for each ORF were selected, separate RT and PCR steps were adopted, and *Taq* Hot Start DNA polymerase (TaKaRa) was used to minimize dimer formation and non-specific amplification. The different RT and PCR efficiency between primer sets was eliminated by carrying out RT-PCR of standard and test samples at the same time, and

variation among reactions was avoided by ROX Reference Dye (TaKaRa) in PCR mixture. Due to these efforts, the established quantification system had a high sensitivity and specificity.

Standard curves indicated that the PCR efficiencies for 1a ORF, 2a ORF, 2b ORF, 3a ORF and CP ORF ($E=10^{-1/slope}-1$) were 125%, 97%, 105%, 98% and 80%, respectively. These values reflected less than optimal PCR conditions for 1a ORF and CP ORF. But this is not entirely unexpected, as the amplification of template by PCR is a process involving multiple components, including structure of target gene, amount of templates, primers, ions, nucleotides, enzyme activity, and reaction temperature. All of them are likely to be dynamically changed as the reaction progresses and to subsequently affect amplification efficiency. The C_t value for the first dilution gradient of 1a ORF was below 10, which is unreliable in data analysis and also will influence the calculation of amplification efficiency. However, it is to be noted that quantification using the standard curve method may be used without detailed optimization [23,24].

To evaluate the efficiency of real-time RT-PCR, the amounts of Fny-CMV genomic RNAs in virions were also determined by Lab-on-a-Chip and Northern blot hybridization. RNA 4A was not detected in virions by all three methods, which was consistent with the reports that RNA 4A could not be encapsidated by strains of CMV subgroup I, or only at very low levels. The copy number ratios determined by three methods were compared, indicating that these methods correlated with each other, except the larger variations of Northern blot hybridization. The results of the Lab-on-a-Chip assay were similar to those of the real-time RT-PCR, but the major constraint of this method was the high purity requirements on test samples. While the levels of viral RNAs in the host were relatively low, it was impossible to study the amounts of viral RNAs in plant tissues by this method directly. Northern blot hybridization also got the ratios closed to those of other methods, but it has larger variations in quantification results resulting from its own defects. For real-time RT-PCR, the amounts of target genes are accurately recorded as C_t values and analyzed in a standard format. This makes the assay considerably less subjective than other methods and more suitable for quantification assay.

In conclusion, the real-time RT-PCR assay presented here offers a sensitive and rapid method for high throughput detection and quantification of Fny-CMV genomic RNAs. To our knowledge, this is the first report for quantification of different RNA fragments in tripartite virus. The accurate quantification property of this assay could be useful to monitor viral replication kinetics, such as the changes of copy number ratios between genomic RNAs in the progress of an infection, the effects of satellite RNA on helper virus, the response to antiviral therapy, and the evaluation of viral tolerance levels in new breeding programs. Northern blot hybridization also got the ratios closed to those of other methods, but it has larger variations in quantification results resulting from its own defects. For real-time RT-PCR, the amounts of target genes are accurately recorded as C_t values and analyzed in a standard format. This makes the assay considerably less subjective than other methods and more suitable for quantification assay.

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