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Research article

Development of a reverse-transcription droplet digital PCR method for quantitative detection of *Cucumber green mottle mosaic virus*

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ABSTRACT

Cucumber green mottle mosaic virus (CGMMV) is a re-emerging threat to the production of greenhouse cucumber and other Cucurbitaceae crops worldwide. This seed-borne virus can easily spread from a contaminated seed to seedlings and adjacent plants by mechanical contact between the foliage of diseased and healthy plants, causing extensive yield losses. An accurate method for detecting and quantifying this virus is urgently needed to ensure the safety of the global seed trade. Here, we report the development of a reverse-transcription droplet digital polymerase chain reaction (RT-ddPCR)-based method for specific and high-sensitive detection of CGMMV. By testing three primer–probe sets and optimizing reaction conditions, we showed that the newly developed RT-ddPCR method is highly specific and sensitive, with a detection limit of 1 fg/μL (0.39 copy/μL). The sensitivity of the RT-ddPCR method was compared with that of real-time fluorescence quantitative RT-PCR (RT-qPCR) using a series of plasmid dilutions and total RNAs extracted from infected cucumber seeds, and the detection limit of RT-ddPCR was 10 times higher than RT-qPCR with plasmid dilutions and 100 times higher than RT-qPCR for detecting CGMMV from infected cucumber seeds. The RT-ddPCR method was further assessed for detecting CGMMV from a total of 323 samples of Cucurbitaceae seeds, seedlings, and fruits as compared with the RT-qPCR method. We found that the infection rate of CGMMV on symptomatic fruits was as high as 100%, whereas infection rates were lower for seeds and lowest for seedlings. Notably, the results of two methods in detecting CGMMV from different cucurbit tissues showed the high consistency with Kappa value from 0.84 to 1.0, demonstrating that the newly developed RT-ddPCR method is highly reliable and practically useful for large-scale CGMMV detection and quantification.

1. Introduction

Cucumber green mottle mosaic virus (CGMMV), a member of the genus *Tobamovirus*, has reemerged as a big threat to the production of greenhouse cucumber and other Cucurbitaceae crops worldwide in recent years. Although CGMMV was firstly found and described in 1935 (Ainsworth 1935), it spread slowly in the subsequent 50 years. However, its global distribution increased more rapidly from

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1986 to 2006, and since 2007, CGMMV has spread quickly and caused varying degrees of disease damages in many countries of Asia, Europe, South America, North America, and Oceania along with the rapid development of international trades (Dombrovsky et al., 2017; Chen and Li, 2007; CABI, 2022). CGMMV mainly infects watermelon, cucumber, pumpkin, and some other cash crops of the Cucurbitaceae family. The fruits of CGMMV-infected watermelon plants show internal flesh sponginess and rotting, and the pulp around the seeds is fibrous and loses its edible value, posing a serious threat to melon production (Dombrovsky et al., 2017). Viral contamination occurs mainly on the surface of the seeds (Faris-Mukhayyish and Makkouk 1983; Liu et al., 2014; Sui et al., 2019), but CGMMV can also be detected in the endosperm envelope beneath the seed coat (Komuro et al., 1971). It was reported that *Tobamovirus* particles, including those of CGMMV, are very stable and their infectivity can persist for several years (Reingold et al., 2016; Dombrovsky et al., 2017; Chanda et al., 2021). In China, CGMMV has been listed as an important import quarantine and national agricultural quarantine pest. Therefore, rapid and reliable detection and diagnosis of virus infection are critical to prevent the spread of this virus and reduce the yield loss. Especially, quantitative viral assays could advance our understanding of CGMMV biology and help to manage viral diseases, whereas such assays are still not available yet for CGMMV and many other plant viruses.

Some approaches based on the biological (Constable et al., 2017), immunological (Takahashi et al., 1989; Kawai et al., 1985), and nucleic acid properties of viruses for the specific detection of CGMMV have been developed in recent years. The most widely used method is conventional reverse transcription (RT)-PCR (Chen et al., 2006; Kan et al., 2010; Zhang et al., 2011) and presently, improved versions of PCR-based assays such as immuno-capture RT-PCR (Shang et al., 2010), recombinase polymerase amplification (RPA) and real-time fluorescence quantitative RT-PCR (RT-qPCR) have been developed for CGMMV detection (Jiao et al., 2019; Chen et al., 2008). However, these reported molecular diagnostic assays can only be used for qualitative detection of CGMMV, including RT-qPCR assay, which can only be considered as a semi-quantitative approach because the cycle threshold (Ct) value of RT-qPCR assay cannot be directly read out as viral loads without the precise calibration to a quantitative standard with known virus titer.

Droplet digital polymerase chain reaction (ddPCR) assay is a newly emerging technique that enables the precise detection and quantification of target DNA/RNA molecules (Kojabad et al., 2021). Like RT-qPCR assay, ddPCR employs target-specific primers coupled with a fluorescent probe, making it relatively straightforward to implement the assay in most of the molecular laboratories. However, in contrast to RT-qPCR assay, in which the whole reaction and PCR amplification occur in one tube, the reaction solution of ddPCR is firstly fractionated into 20,000 nL-sized droplets and then these droplets undergo massively parallel PCR amplifications. Finally, each droplet is categorized as positive or negative based on the presence or absence of the target DNA/RNA, allowing for absolute quantification of the targets using Poisson statistics (Vogelstein and Kinzler Kinzler, 1999; Pinheiro et al., 2012). This sensitive and versatile technology has been used for mutation detection and copy number determination in the human genome (Salemi et al., 2018), target verification following genome editing (Findlay et al., 2016), and quantification of viral pathogens (Kinloch et al., 2021; Larsson and Helenius, 2017; Pandey et al., 2020; Persson et al., 2018; Kojabad et al., 2021). Despite these reports, few studies have been reported to use the RT-ddPCR method for the detection of plant viruses. In this study, we developed a quantitative RT-ddPCR method for high-sensitive detection of CGMMV and performed a comprehensive comparison between RT-ddPCR and RT-qPCR assays.

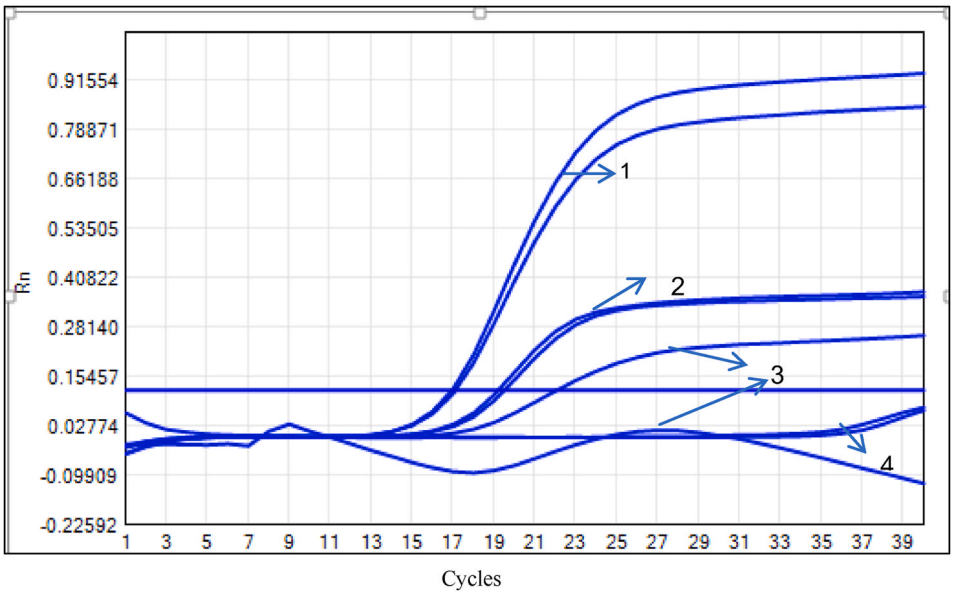

Figure 1. Amplification curves of one-step RT-qPCR using different primer–probe combinations.
Three primer-probe sets (1, CGMMV-114F/R/P; 2, CGMMV-97F/R/P; 3, CGMMV-79F/R/P) and total RNAs extracted from CGMMV-infected cucumber leaves as the initial template were used for one-step RT-qPCR amplification. Only the RT-qPCR assay with the primer-probe set of CGMMV-114F/R/P but lacking the RNA template was used as the control (4). Two technical replicates for each primer-probe set were performed in RT-qPCR assays.

2. Results

2.1. Screening of CGMMV-specific primers and probes for RT-qPCR and RT-ddPCR detection

Three sets of primers and probes, CGMMV-114F/R/P, CGMMV-97F/R/P, and CGMMV-79F/R/P, were designed based on the reported CGMMV sequences using the GenScript real-time PCR (Taqman) primer design tool. We first used one-step RT-qPCR to test the performance of these three primer-probe sets using total RNAs extracted from CGMMV-infected cucumber leaves as the initial template. The one-step RT-qPCR tests were replicated twice for each primer-probe set. As shown in Figure 1, compared with the other two primer-probe sets, the primer-probe combination CGMMV-114F/R/P rendered the RT-qPCR amplification with much earlier presence of the peaks and stronger fluorescence signals, indicating that this primer-probe set has the best performance among the three primer-probe sets. Therefore, the primer-probe combination CGMMV-114F/R/P was then used in the subsequent experiments.

2.2. Optimization of reverse transcription reaction temperature

The efficiency of RT reaction using specific primers is affected by the reaction temperature. Given that the costs of one-step RT-qPCR and RT-ddPCR assays for large-scale detection are much higher than their two-step counterparts, we therefore decided to develop two-step RT-ddPCR method with separate RT and ddPCR reactions. To obtain an optimized RT temperate, based on the temperature range recommended in the RT kit instruction (42–55 °C), we tested six different temperatures (42 °C, 45 °C, 48 °C, 50 °C, 52 °C, and 55 °C) for RT reactions using the same amount of total RNAs extracted from CGMMV-infected cucumber leaves, with each temperature having three biological replicates for RT and three technical replicates for ddPCR. Among the tested temperatures, RT reaction at 48 °C for RT-ddPCR exhibited the highest amplification efficiency, producing nearly 2–5 folds of PCR products compared with RT actions at other temperatures (Figure 2). We therefore selected 48 °C as the optimal temperature for RT reaction in subsequent RT-qPCR and RT-ddPCR assays. The other reaction conditions for RT-qPCR and RT-ddPCR were conventional and are described in the Methods section.

2.3. Specificity of RT-ddPCR

To test the specificity of the RT-ddPCR method, total RNAs were extracted from the samples infected with seven viruses (*Citrullus lanatus* cryptic virus, CiLCV; *Watermelon mosaic virus*, WMV; *Watermelon crinkle leaf-associated virus* 1, WCLaV-1; *Zucchini yellow mosaic virus*, ZYMV; and *Melon aphid-borne yellows virus*, MABYV) commonly carried by melon seeds, respectively. The extracted RNAs were then used as the templates for RT reactions and then RT-ddPCR assays were performed using the CGMMV114 F/R/P primer-probe set. As shown in the graph of Figure 3, the specific droplet amplification signals (blue dots) were observed only in the CGMMV-infected sample, and there were no droplet signals in the samples infected with the other six viruses or in the healthy control. Two independent biological replicates for RT reactions and three technical replicates for ddPCR assays using the same primer-probe sets were performed with similar results, indicating that the RT-ddPCR method has the high specificity for CGMMV detection.

2.4. Sensitivity of RT-ddPCR

To determine the sensitivity of the RT-ddPCR method for CGMMV detection, the 100 ng/μL stock solution of the plasmid carrying the CGMMV CP gene was 10-fold diluted sequentially with TE buffer to create a dilution series (from 100 ng/μL to 10 ag/μL). This series of the dilutions were then used separately as the templates for ddPCR amplification. All dilutions higher than 1 fg/μL (A03) gave

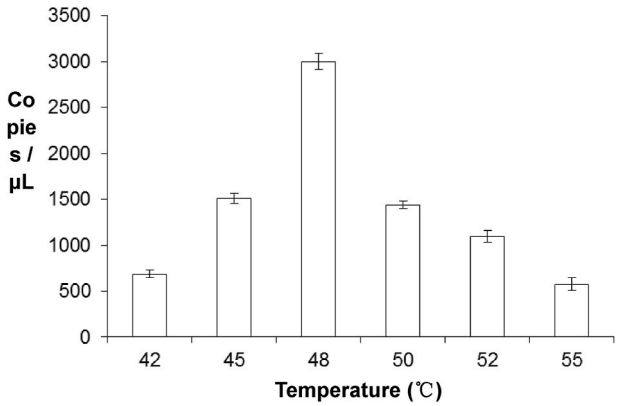

Figure 2. Optimization of RT reaction temperatures for RT-ddPCR.
RT reactions were performed at six different temperatures (42 °C, 45 °C, 48 °C, 50 °C, 52 °C, and 55 °C) using the same amount of total RNAs extracted from CGMMV-infected cucumber leaves and then ddPCR assays with three replicates were carried out using 2μL RT products.

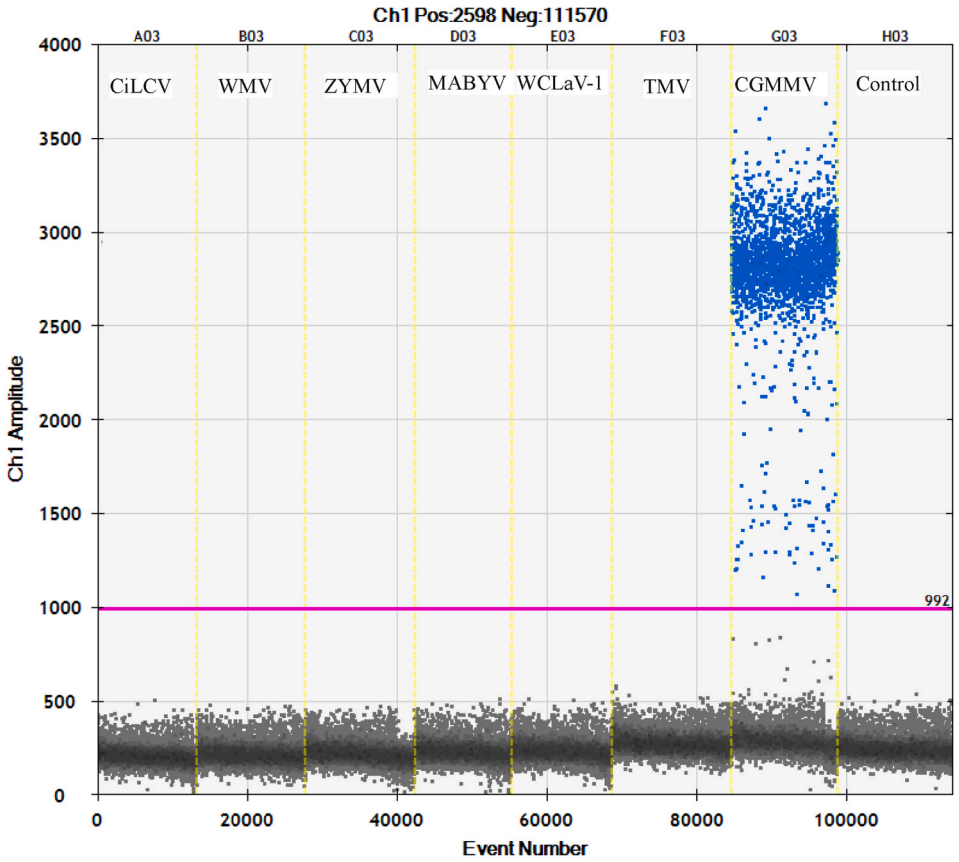

Figure 3. Specificity of the RT-ddPCR method using the CGMMV114 F/R/P primer–probe set for CGMMV detection.
RT-ddPCR assays were performed using the CGMMV114 F/R/P primer–probe set and total RNAs extracted from the samples infected with CGMMV and six other viruses, respectively. Two independent biological replicates for RT reactions and three technical replicates for ddPCR assays using the same primer-probe sets were performed with similar results. A03, CiLCV; B03, WMV; C03, ZYMV; D03, MABYV; E03, WCLaV-1; F03, TMV; G03, CGMMV; H03, blank control.

rise to strong amplification signals (blue dots) and more than 2 blue dots (5 ± 3) of amplification signals could be consistently seen for the dilution 1 fg/μL (A03) in four independent experiments. A representative ddPCR droplet distribution graph is provided in Figure 4. Usually, for a given sample, if more than 2 copies in a 20μL reaction system are detected by RT-ddPCR, this sample is considered as positive (Dreo et al., 2014). Therefore, our results indicate that the limit of ddPCR is the dilution of 1 fg/μL (Table 2), corresponding to 0.39 copy/μL or 7–8 copies of template in 20μL reaction system.

2.5. Reproducibility of RT-ddPCR

To validate the reproducibility of RT-ddPCR, five dilutions (0.1 ng/μL, 0.05 ng/μL, 0.01 ng/μL, 0.001 ng/μL and 0.00002 ng/μL) of the plasmid carrying the CGMMV CP gene were generated and ddPCR assays were performed with eight replicates for each dilution. As shown in Table 1, the coefficient of variation (CV) of ddPCR varied for each dilution, with the lowest CV that was observed for the dilution of 0.01 ng/μL, corresponding to 492.13 copies/μL of the template. Among the five plasmid dilutions, the ddPCR assay using the lowest one (0.00002 ng/μL) produced the highest CV (22.24%), indicating that ddPCR results at this concentration (less than 2 copies/μL of the template) are not very reliable and should be verified by multiple repeated tests.

2.6. Comparison of RT-ddPCR with RT-qPCR

To compare the sensitivity of RT-ddPCR and RT-qPCR for CGMMV detection, a series of ten-fold dilutions of the plasmids carrying the CGMMV CP gene was generated and used as the templates for PCR amplification. As shown in Table 2, RT-ddPCR could detect more than 2 copy of the template from the dilution as low as 1 fg/μL per 20 μL reaction and those higher than 1 fg/μL, while RT-qPCR could produce the positive amplification signals with the Ct value lower than 35 cycles from the dilution 10 fg/μL and higher. Three independent replicates for RT-ddPCR and RT-qPCR were performed and similar results were obtained, whereas there are some slight variations in each replicate, indicating that RT-ddPCR has 10 times higher sensitivity than RT-qPCR using the plasmid as the template.

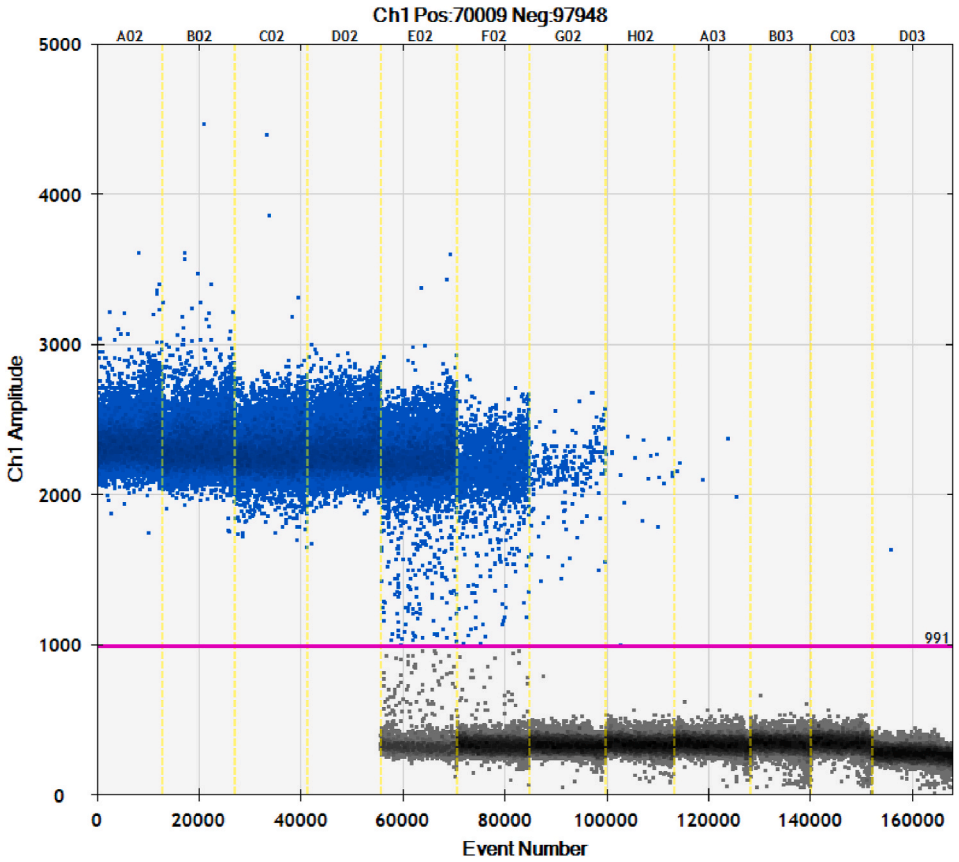

Figure 4. Sensitivity of the ddPCR method assessed using a series of plasmid dilutions.
A02, 100 ng/μL plasmid stock solution; B02, 10 ng/μL dilution; C02, 1 ng/μL dilution; D02, 100 pg/μL dilution; E02, 10 pg/μL dilution; F02, 1 pg/μL dilution; G02, 100 fg/μL dilution; H02 10 fg/μL dilution; A03 1 fg/μL dilution; B03, 100 ag/μL dilution; C03, 100 ag/μL dilution; D03, No template control. A threshold discrimination (pink line) between droplets that did not contain the target (negatives) and those that did (positives) was based on the signals read by the QuantaSoft software (Bio- Rad).

Table 1
Reproducibility of the RT-ddPCR method.

No.	Mean (ng/μL)	SD*	CV** (%)	Copies/μL
1	0.1	224.53	6.14	5285
2	0.05	113.53	5.62	2484.88
3	0.01	12.31	3.90	492.13
4	0.001	3.31	5.9	56
5	0.00002	0.24	22.24	1.78

*, Standard deviation; **, Co-efficient of variation.

To further compare the sensitivity of RT-ddPCR and RT-qPCR for detecting CGMMV from the infected samples, total RNAs were extracted from a CGMMV-infected cucumber seed sample and a series of total RNA dilutions was used for reverse transcription to synthesize cDNA templates for both RT-ddPCR and RT-qPCR assays. Three independent replicates of RT-ddPCR and RT-qPCR assays were performed for detecting CGMMV from the infected samples and similar results were obtained, whereas there are some slight variations in each replicate. The results showed that RT-ddPCR could detect more than 2 copy of CGMMV from the dilution as low as

Table 2
Comparison of the detection limits of ddPCR and qPCR methods assessed using the plasmid dilutions.

	1 ng/μL	100 pg/μL	10 pg/μL	1 pg/μL	100 fg/μL	10 fg/μL	1 fg/μL	100 ag/μL
ddPCR (copies/μL)	No call	5150 ± 198	1397 ± 23	139 ± 6	12.3 ± 1.8	1.12 ± 0.15	0.27 ± 0.04	0
qPCR (Ct)	17.52 ± 1.08	20.28 ± 0.43	24.83 ± 0.21	28.95 ± 0.35	31.61 ± 0.28	34.71 + 0.12	38.12 ± 0.34	39.86 ± 0.05

No call, the number of positive droplets in the sample was too large to count.

Table 3
Detection limits of RT-ddPCR and RT-qPCR assessed using total RNAs extracted from infected seeds.

	RNA						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
RT-ddPCR (copies/μL)	641 ± 21	55.7 ± 4.8	7.7 ± 1.7	3 ± 0.9	0.21 ± 0.04	0.13 ± 0.02	0
qPCR (Ct)	26.04 ± 1.01	29.27 ± 1.23	33.13 ± 0.67	37.75 ± 1.31	No Ct	No Ct	No Ct

10^{-5}, while RT-qPCR could detect CGMMV from the dilution as low as 10^{-3} with the Ct value lower than 35 cycles (Table 3). These results suggest that the detection sensitivity of the RT-ddPCR method is 100 times higher than that of RT-qPCR for detecting CGMMV from the infected seed samples.

2.7. Application of RT-ddPCR to detect CGMMV from cucurbit samples

We collected 89 samples of cucurbit seeds (including watermelon, melon, cucumber, and pumpkin) imported from other countries at the Shanghai Port, and 234 samples of seeds (96), seedlings (128), and fruits (10) of cucurbit species from the markets or cucurbit-planting fields in Shanghai, Jiangsu, and Ningxia, China. The 10 fruit samples collected from cucumber and watermelon fruits that exhibited viral disease symptoms. We then used both RT-ddPCR and RT-qPCR to detect the presence of CGMMV in these samples (Table 4; a total of 323 samples, each sample with two technical replicates). We found that CGMMV could be detected by RT-ddPCR in 33 seed samples (from a total of 185 seed samples), 5 seedling samples, and 10 fruit samples, whereas RT-qPCR could detect the presence of CGMMV from all 10 fruit samples and 6 seedling samples, but only 30 seed samples could be detected with the presence of CGMMV by RT-qPCR. We used the Kappa value to evaluate the consistency of the two methods for CGMMV detection and found that the Kappa value between the two methods was >0.84 for total samples, indicating that RT-ddPCR could accurately detect CGMMV in the infected samples. Interestingly, for the seed samples, the positive rate of RT-ddPCR was slightly higher than that of RT-qPCR (Table 4). When the RT-ddPCR results of the positive seed samples were quantified, the concentration of CGMMV in the infected seeds ranged from 1.2 copies/μL to 10,000 copies/μL.

3. Discussion

Quantitative detection of pathogenic agents is important for human disease diagnosis and ensuring environmental and food security. Although many molecular methods have been developed for the detection of plant viruses, most of them cannot be easily used to quantify the precise viral titers from the infected plant tissues. Even for RT-qPCR, the precise quantification of the titer of a certain virus in the infected plant tissues requires the standard virus sample with known titer and time-consuming correlative mathematic calculation. Due to the lack of the standard virus samples for the majority of plant viruses, the precise quantification methods are not available for the majority of plant viruses in most common laboratories, dampening the quantitative detection of plant viruses and thus increasing the risk of their spread and outbreaks of in the fields. Breeding and cultivation of the virus-resistant varieties are critical for controlling plant viral disease (Hajano et al., 2016); however, selection of virus-resistant varieties during breeding programs also requires the straightforward quantitative methods for detecting the viruses and evaluating the virus resistance of breeding materials (Picó et al., 2005).

In this study, we developed a high-sensitive quantitative RT-ddPCR method for CGMMV detection. Like RT-qPCR, the RT-ddPCR assay is also a high-throughput method with a streamlined procedure for virus detection, as it can also be performed simultaneously in the 96-well plates, with the results that can be rapidly read out via the QX200 droplet reader. Importantly, different from RT-qPCR, RT-ddPCR can precisely quantify the viral titer without the need of the standard virus sample and complicated correlative calculation, making the quantification of viral titers in plant tissues much feasible. Using plant issues infected by CGMMV or six other Cucurbitaceae viruses, we showed that the RT-ddPCR method we established has the high specificity and can only detect the target virus (CGMMV) from the infected samples. We further compared the sensitivity of RT-qPCR and RT-ddPCR assays, and showed that RT-ddPCR is more sensitive than RT-qPCR for CGMMV detection. The highly sensitive and specific RT-ddPCR method we developed is very useful for rapid detection of CGMMV from the seeds of cucurbit plants, which are often being transported for long distance along the international trades. Despite the above-mentioned advantages, compared with the RT-qPCR assay, the RT-ddPCR method currently suffers from some limitations, including 1) each reaction of RT-ddPCR is about 5–10 fold expensive than that of RT-qPCR, 2) it requires some special devices such as the QX200 droplet generator and reader for implementing the assay, and 3) it is a bit time-consuming and typically requires one more hour to obtain the assay result. To make the RT-ddPCR method become a routine tool for plant virus detection, these limitations should be overcome in the future.

Infection of cucurbit seeds by CGMMV is very common. The CGMMV infection rate on watermelon seeds could reach 100%, whereas the rate of its transmission to the progenies via seeds is only 2.25%, as determined by planting infected seeds and detecting the seedling with RT-PCR (Wu et al., 2011). The CGMMV infection rates in melon and gourd seeds were reported to be 93.85% and 100%, respectively, whereas its transmission rates in the seeds of these two cucurbits are 2.83% and 1.01%, respectively (Qin et al., 2011). Virus-containing seeds are the main source of initial CGMMV infection in the fields. The RT-ddPCR method we reported here provides a powerful tool for CGMMV detection from the seeds and other infected plant tissues, which will reduce the spread and outbreak of this virus in many countries and regions. Moreover, seed treatment with chemical reagents or heat is the commonly used strategy for

Table 4
Application of RT-ddPCR and RT-qPCR for detecting CGMMV from cucurbit seeds, seedlings, and fruits.

Sample	Number	RT-ddPCR		Real time RT-PCR		Kappa value
		Positive	Negative	Positive	Negative	
Seeds	185	33	152	30	155	0.89
Seedlings	128	5	123	6	122	0.84
Fruits	10	10	0	10	0	1
Total	323	48	275	46	277	0.95

eliminating seed surface-contaminated viruses at the ports when seeds are imported from or exported to other countries and regions (Kim et al., 2003; Kim and Lee, 2000) . However, the viruses may not be completely cleaned up by such treatments because some viruses may be very stable when they infect the embryos in the seeds or high levels of viruses are carried by the seeds. Employment of RT-ddPCR method to quantitatively detect the viruses from the seeds after the treatment will enable the evaluation of the effectiveness of the treatments and ensure the release of only virus-free seeds for the trades.

In summary, the RT-ddPCR method we developed in this study is highly sensitive and specific for quantitative detection of CGMMV from the seeds and other plant tissues. This method will not only be useful for the detection of CGMMV in the virus diagnostic laboratory but also might be applicable for fundamental research of CGMMV biology and assisting the breeding of virus-resistant crops. Our work also provides a case study on the development of RT-ddPCR methods for the detection of other plant viruses.

4. Materials and methods

4.1. Plant materials and viruses

The cucumber seeds and freeze-dried leaves infected with CGMMV, *Citrullus lanatus* cryptic virus (CiLCV), *Watermelon mosaic virus* (WMV) *Watermelon crinkle leaf-associated virus 1* (WCLaV-1) *Zucchini yellow mosaic virus* (ZYMV), and *Melon aphid-borne yellows virus* (MABYV) were intercepted from imported cucumber, watermelon and melon seeds, and *Tobacco mosaic virus* (TMV) are stocks maintained in our laboratory.

4.2. Primers and probes

Three sets of primers and probes targeting the conserved domain of coat protein and movement protein genes of CGMMV were designed based on the sequences obtained from the NCBI GeneBank database (accession numbers shown below) and the GenScript real-time PCR (Taqman) primer design tool. They were used to develop the RT-ddPCR detection method. All primers and probes were synthesized by BGI Biotechnology, Ltd., and their sequences are shown in Table 5.

4.3. Plasmid construction

The CGMMV CP was amplified using the primer pair CGMMV-194F (5'- GTTTTCGGTAGTCTGGTCAGAGGCTA) and CGMMV-194R (5'-GGATTCGAACCCCTTGCAGAATTAC) and cloned into the pMD TM 18-*T vector* (Takara, Dalian, China). The plasmids carrying CGMMV CP gene were multiplied in *E. coli* DH5α strain and purified using the Plasmid DNA Mini Kit I (Omega, USA) according to the manufacture's instructions.

4.4. RNA extraction

Seeds and leaves were ground in a freeze-mill or mortar, respectively, and total RNAs were extracted using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen, China) according to the manufacture's instructions. The RNA quality was determined by the Qubit assays on a Qubit 4 Fluorometer (ThermoFisher, USA) according to the manufacture's instructions.

Table 5
CGMMV primer-probe sets assessed for use in RT-ddPCR.

Primer/probe	Sequence (5′–3′)	Target gene
CGMMV-114F	GCATAGTGCTTTCCCGTTCAC	Coat protein
CGMMV-114R	TGCAGAATTACTGCCCATAGAAAC	
CGMMV-114P	FAM-CGGTTTGCTCATTGGTTTGCGGA-BHQ	
CGMMV-79F	TACGCTTTCCTCAACGGTCC	Coat protein
CGMMV-79R	TGACCCTATTACGCGTATCCGT	
CGMMV-79P	FAM-ATCTTCGTTTCGCTTCT-MGB	
CGMMV-97F	TGATCTTACAAAACACCTTTATGTCACA	Movement protein
CGMMV-97R	AGCAACAGTAGCACCACCCCT	
CGMMV-97P	FAM-CATACACCAGAAACCACAACGCCCAAC-Tamra	

4.5. RT-ddPCR

The RT-ddPCR reaction was performed on a QX200 Droplet Digital PCR System (Bio-Rad, USA). It consists of five steps: first-strand cDNA synthesis, system formulation, droplet generation, amplification, and signal reading. According to the instructions of the Droplet PCR Supermix kit (Bio-Rad, USA), reaction systems containing 4.0 μL 5× PrimeScript IV cDNA Synthesis Mix, 0.2 μL 3'-terminal specific primers (final concentration 0.1 μM), 2.0 μL RNA template, and ddH_2O to 20 μL were prepared in sample tubes. The reaction systems were incubated at 42–54 °C for 15 min. The reverse transcriptase was inactivated at 70 °C for 15 min, and the cDNA product was then stored at −20 °C for later use. Each designated well of a DG8 cartridge assembled with the DG8 cartridge holder was manually loaded with 20 μL of reaction mixture and 70 μL of QX 200 droplet generation oil and placed in the QX 200 droplet generator (Bio-Rad, USA) for droplet creation. The droplets were then transferred to a 96-well PCR plate. The amplification cycles were as follows: 94 °C for 10 min; 40 cycles of 94 °C for 15 s and 60 °C for 60 s; 98 °C for 10 min; and holding at 12 °C. The amplified 96-well plate was placed in a QX200 droplet reader (Bio-Rad, USA) for signal reading, and the test data were analyzed using Quanta Soft V1.3.2 software (Bio-Rad, USA).

4.6. RT-qPCR

RT-qPCR was performed on a GNCycler-96s real-time fluorescence instrument (Jienuo Technology Co. Ltd., Shanghai, China).With Probe qPCR mix kit (Takara, China). Each reaction mixture contained 10 μL 2×probe q PCR mix, 0.4 μL each forward and reverse primers (10 μM), 0.3 μL probe (10 μM), 2 μL template, and RNase-free ddH_2O to 20 μL. The reaction conditions were 50 °C for 30 min, 95 °C for 10 sec, and 40 cycles of 95 °C for 10 sec and 60 °C for 40 sec.

4.7. Accession numbers

The accession numbers of the following complete genome sequences of CGMMV isolates are used in this study: D12505, KR232571, KU140423, KU140425, KX443591, KX443593, KX555505, KY753927, KY910828, MF510469, MG745849, MH271439, MH271443, MH426842, MK 933286, NC-001801.

Declarations

Author contribution statement

Cui Yu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Yimin Tian: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Jing Fei: Analyzed and interpreted the data; Wrote the paper.
Jinyan Luo, Lei Chen, Jun Ye and Wei Du: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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