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Development and Application of Reverse Transcription Nanoplate-Based Digital PCR Assay for Sensitive and Accurate Detection of Rice Black-Streaked Dwarf Virus in Cereal Crops

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The emergence of rice black-streaked dwarf virus (RBSDV) poses a significant threat to global cereal crop cultivation, necessitating the urgent development of reliable detection and quantification techniques. This study introduces a reliable approach for the precise and sensitive quantification of the RBSDV in cereal crop samples, employing a reverse transcription digital polymerase chain reaction (RT-dPCR) assay. We assessed the specificity and sensitivity of the RT-dPCR assay proposed for precise RBSDV detection and quantification. Our findings demonstrate that RT-dPCR was specific for detection of RBSDV, with no cross-reactivity observed with other viruses infecting cereal crops. The RT-dPCR sensitivity was over 10 times that of RT-quantitative PCR (RT-qPCR). The detection limit of RT-dPCR was 0.096 copies/µl. In addition, evaluation of RT-dPCR assay with field samples was conducted on 60 different cereal crop samples revealed that RT-dPCR (45/60) exhibited superior accuracy compared with RT-qPCR (23/60). In this study, we present a specific and accurate RT-dPCR assay for the detection and quantification of RBSDV.

Keywords **:** detection, reverse transcription digital PCR, rice black-streaked dwarf virus

Cereal crops, including barley, wheat, and oats, are fundamental to global food security, providing sustenance for billions of people worldwide. However, the productivity and yield of these crops are continually threatened by various viral pathogens, among which rice black-streaked dwarf virus (RBSDV) poses a significant challenge (Wu et al., 2020). RBSDV, a member of the genus *Fijivirus* within the family *Reoviridae*, is transmitted by the small brown planthopper (*Laodelphan striatellus*) and can inflict severe damage to cereal crops (Wang et al., 2022). RBSDV exhibits distinct biological characteristics that contribute to its pathogenicity and spread. It possesses a segmented double-stranded RNA genome comprising 10 segments, each encoding specific viral proteins essential for viral replication, transcription, and transmission. The virus primarily infects the phloem tissues of the host plants, leading to systemic spread and characteristic symptoms such as stunted growth, leaf chlorosis, and the formation of black streaks on stems and leaves. These symptoms impair photosynthesis and nutrient transport and render infected plants more susceptible to secondary infections and environmental stresses, exacerbating yield losses in affected crops. In recent years, RBSDV outbreaks have been reported across various regions of Korea, posing a significant threat to cereal crop cultivation and food security (Lee et al., 2005). Moreover, RBSDV has been newly reported in oats in Korea by metatranscriptomics (Kim et al., 2022a).

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Despite considerable efforts to combat RBSDV, its efficient detection remains a critical bottleneck in disease management strategies. Currently, conventional molecular detection methods, such as RT-PCR and TaqMan-probed real-time quantitative PCR (RT-qPCR), have been widely utilized (Kim et al., 2009; Zhang et al., 2013). Additionally, various isothermal amplification techniques have recently been used to detect RBSDV (Hua et al., 2023; Zhao et al., 2019). However, these methods often exhibit sensitivity, specificity, and quantification accuracy limitations. Hence, these challenges underscore the urgent need for innovative diagnostic tools to reliably detect RBSDV in cereal crops with high sensitivity and precision.

Owing to this need, herein, we present the development and application of a digital PCR (dPCR) assay tailored for a specific and sensitive RBSDV in cereal crops. dPCR assay has been employed to detect several plant viruses in various crops (Kim et al., 2022b, 2023). dPCR offers several advantages over conventional PCR methods, including absolute quantification without reliance on standard curves, enhanced sensitivity, and robustness against inhibitors. By partitioning the PCR reaction into thousands of individual reactions, dPCR enables precise quantification of target nucleic acids, even at low concentrations, making it an ideal choice for virus detection in viral disease management (Lei et al., 2021). Nanoplate-based dPCR partitions the sample into individual reaction wells, usually on a microfluidic chip, with each well holding a small volume of the sample mixture. In addition, fluorescent probes or dyes are used to detect amplification products within each reaction well, with the fluorescent signal measured individually for each well. Both nanoplate-based dPCR and regular dPCR are used for similar applications. The choice between the two techniques depends on factors such as throughput requirements, ease of use, and budget constraints (Lei et al., 2021).

In this study, we outline the experimental procedures employed for assay development, including primer and probe, optimization of reaction conditions, testing of specificity to other cereal viruses, evaluation of sensitivity with TaqMan-probed RT-qPCR, and finally, validation using field-collected cereal crop samples.

RBSDV-infected oat (*Avena sativa*) leaves showing leaf chlorosis, mild black streaks on the leaves, and stunted growth along with RBSDV-free leaves were provided by the National Institute of Crop Science of Korea. The samples were kept in a deep freezer at –80°C. Total RNA extraction was performed utilizing the Clear-S Total RNA extraction kit (InVirus Tech Co., Gwangju, Korea), following the manufacturer's protocols. RT-PCR was conducted with established diagnostic primers and procedures to test the presence of RBSDV in symptomatic oats leaves (Lee et al., 2005). RT-qPCR and RT-dPCR experiments were conducted using RBSDV-positive RNA samples as templates, with RNA extracted from healthy oat samples as the negative control. The partial coat protein (CP) gene of RBSDV, approximately 501 bp in length, amplified by RT-PCR, was cloned using a T&A cloning kit (Yeastern Biotech, Taiwan), followed by obtaining the sequence. The primers PBSDV-F1 (5′-CTGTTTACAAAACCGATCCA-3′) and RBSDV-R1 (5′-CGGAAAGTGCCTTCTTTAAT-3′), along with the probe (6-FAM-CATCTAGTCATGAA-CATGTACAGAAGATAGTA-BHQ-1), were designed using Primer 5.0 software based on highly conserved sequence identified in BioEdit version 7.0.5.3 of the CP gene from 100 isolates obtained from the GenBank (Supplementary Fig. 1). These primers and the probe were synthesized by Bionics (Daejeon, Korea) for subsequent TaqMan RT-qPCR and RT-dPCR analysis. *In vitro* transcription of RBSDV-CP was performed as described by Lee et al. (2021). The concentrations of the synthesized RNA transcripts were assessed using a BioDrop spectrophotometer (Biochrom, Ltd., Cambridge, UK). The QIAcuity dPCR reaction was performed using the QIAcuity One 2-plex digital PCR system (Qiagen, Hilden, Germany) by the same operator using cDNA generated from the RT reaction using M-MLV reverse transcriptase (Promega, Madison, WI, USA). The reaction mixture was composed as follows according to the manufacturer's instructions: 10 µl of 4× concentrated QIAcuity Probe Master mix (Qiagen), 900 nM forward and reverse primers and 250 nM probe, and cDNA template in a final volume of 40 µl. The prepared reaction mixture was transferred to QIAcuity 26k 24-well Nanoplates (Qiagen) for partitioning using the Qiagen standard priming profile. The QIAcuity dPCR cycling conditions comprised enzyme activation at 95°C for 2 min followed by 45 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 30 s. Partitions were imaged with 500 ms (FAM channel) exposure time. Data were analyzed using the QIAcuity Suite Software V2.0.20, and the DNA amount was exported as copies/µl.

Various temperature conditions ranging from 56°C to 60°C were tested to optimize the annealing temperature in RT-dPCR. Subsequently, the signal separation between the positive and negative partitions was enhanced when the annealing temperature was reduced, whereas increasing it diminished this separation. Thus, an annealing temperature of 60°C was selected for the RT-dPCR experiment (Fig. 1A). The specificity and cross-reactivity of the RT-dPCR were evaluated for three major cereal viral pathogens in Korea: barley yellow dwarf virus (BYDV), barley mild mosaic

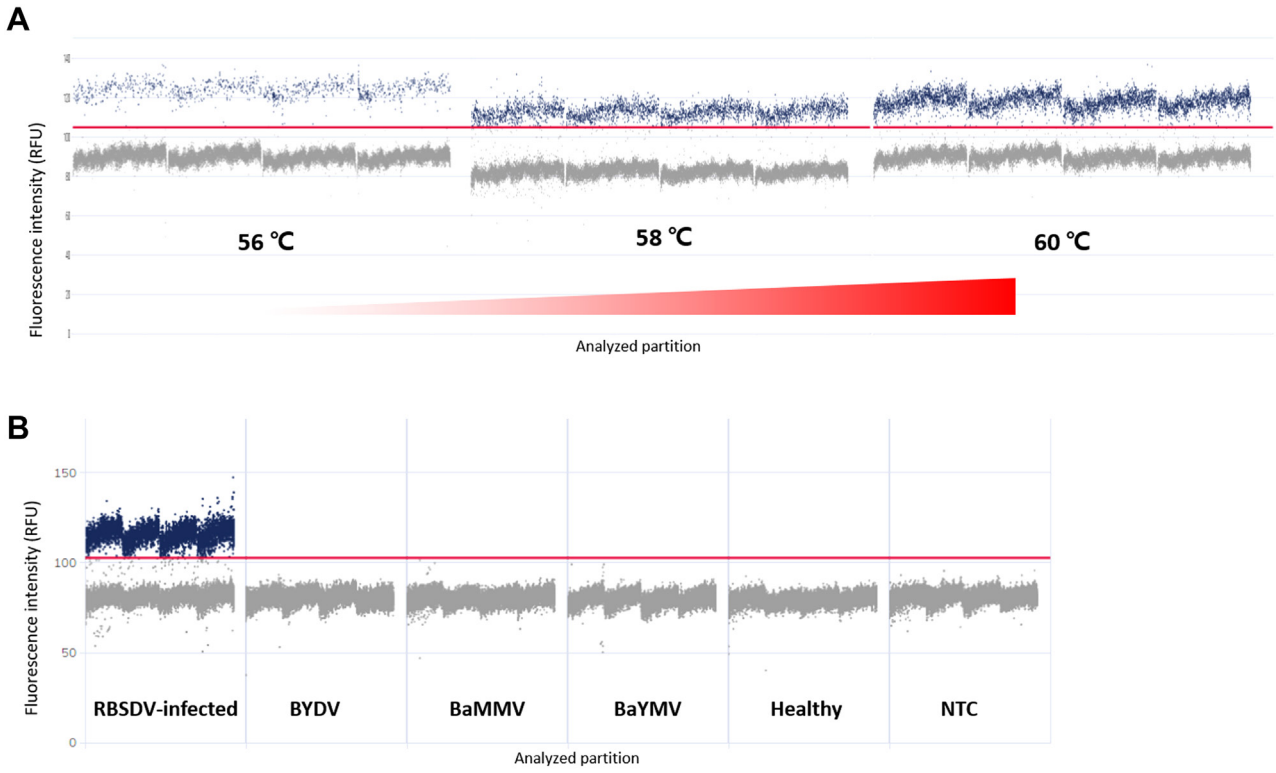

Fig. 1. Optimization of the annealing temperature and evaluation of specificity and cross-reactivity of the reverse transcription digital PCR assay for rice black-streaked dwarf virus (RBSDV) detection. (A) The annealing temperatures for the primers and probe were optimized within the range of 56°C, 58°C, and 60°C. (B) The fluorescence amplitude of barley yellow dwarf virus (BYDV), barley mild mosaic virus (BaMMV), and barley yellow mosaic virus (BaYMV). NTC, non-template control.

virus (BaMMV), and barley yellow mosaic virus (BaYMV). Healthy samples and non-template control (distilled water) were used as the negative controls. Positive signals were detected only in RBSDV-infected samples, while negative signals were observed in other virus-infected samples and the negative controls, indicating high specificity for RBSDV with no cross-reaction (Fig. 1B). The specificity assay for each sample was performed independently three times.

RT-qPCR and RT-dPCR sensitivities were compared using 10-fold serial dilutions (ranging from 3.7×10^8 to 3.7×10^2 copies/µl) of RBSDV-CP transcripts. RT-qPCR reaction was employed EzAmp HS One-step RT-qPCR Master Mix (ELPIS-Biotech, Daejeon, Korea), following the reaction mixture and procedure presented by Lee et al. (2021). A standard curve was generated from *Cq* values obtained from 10-fold serial dilutions of RNA transcripts. The copy numbers were calculated using the following formula: concentration of transcripts (copies/µl) = concentration (µg/µl)/(fragment size [bp] × 182.5 × 10^{13}) (Lee et al., 2021). This assay was conducted in triplicate within a single run and replicated across three independent runs. The lowest detectable concentration for RT-dPCR was 0.096 copies/µl, whereas RT-qPCR detected 1.3 copies/µl. Subsequently, RT-dPCR demonstrated the ability to detect RBSDV at significantly lower concentrations than RT-qPCR. Overall, the sensitivity of RT-dPCR was approximately 100-fold higher than RT-qPCR (Table 1). Both assays exhibited good repeatability, as evidenced by their coefficients of variation. These coefficients of variation s were determined by dividing the standard deviation by the mean of three replicates to assess technical repeatability. Next, the linearity of both methods within the detection range was evaluated using a standard dilution. The results from both RT-qPCR and RT-dPCR demonstrated significant linearity, with R^2 values of 0.9881 and 0.9982, respectively, close to 1 (Fig. 2).

To further validate and compare with RT-qPCR, 60 field leaf samples (barley, oat, and wheat) were randomly collected from three regions (Gwangju, Haenam, and Kangjin) for infection testing and RBSDV quantification using the developed RT-dPCR method. For barley, RBSDV was detected by RT-dPCR in 6 out of 20 samples, whereas RT-qPCR detected no RBSDV in any samples. For oat, RB-

Table 1. Sensitivities of the qPCR and dPCR assays for RBSDV

Sample name	Concentration of cDNA (copies/μl)	qPCR					dPCR				
		Concentration			Mean ± SD	CV (%)	Concentration			Mean ± SD	CV (%)
NTC	-	N/A	N/A	N/A	-	-	0	0	0	-	-
10^{-5}	3.7×10^{8}	14,880.2	13,383.7	12,943.1	13,735.6 ± 1015.3	7.39	8,053.8	10,170.6	12,351.3	10,191.9 ± 2,148.8	11.08
10^{-5}	3.7×10^{7}	1,039.5	817.7	795.2	884.1 ± 135	15.27	1,691.8	1,618.1	1,704.9	1,671.6 ± 46.7	2.8
10^{-6}	3.7×10^{6}	193.8	155.1	134.1	161 ± 30.3	18.81	171.1	164.4	165.7	167 ± 3.5	2.13
10^{-7}	3.7×10^{5}	30.2	29.9	29.9	30 ± 0.2	0.58	18.8	19.3	21.3	19.8 ± 1.3	6.68
10^{-8}	3.7×10^{4}	1.3	1.6	1.8	1.56 ± 0.25	16.06	1.5	2.0	2.1	1.86 ± 0.32	17.22
10^{-9}	3.7×10^{3}	N/A	N/A	N/A	-	-	0.096	0.143	0.288	0.175 ± 0.1	16.97
10^{-10}	3.7×10^{2}	N/A	N/A	N/A	-	-	0.096	0	0.096	-	-

qPCR, quantitative PCR; dPCR, digital PCR; RBSDV, rice black-streaked dwarf virus; SD, standard deviation; CV, coefficient of variation; NTC, non-template control; N/A, not available.

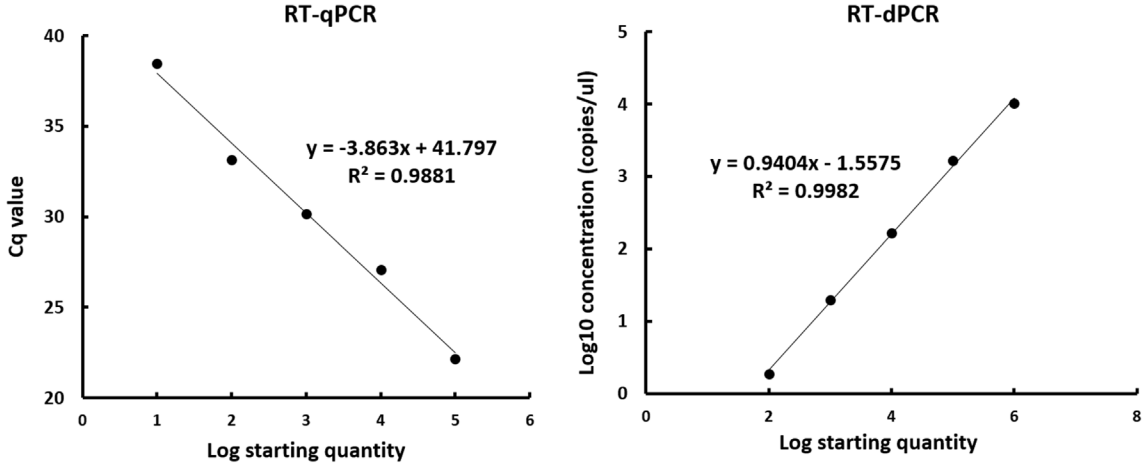

Fig. 2. Linear regression analysis of real-time quantitative PCR (RT-qPCR) and reverse transcription digital PCR (RT-dPCR) comparison. Ten-fold serial dilution of the rice black-streaked dwarf virus (RBSDV)-coat protein (CP) transcripts was employed to assess the correlation coefficients between RT-qPCR (A) and RT-dPCR (B). The quantitative linearity (R^2) correlation coefficients for RT-qPCR and RT-dPCR were 0.9981 and 0.9982, respectively.

SDV was detected by RT-dPCR in 13 out of 20 samples, while RT-qPCR detected it in 8 out of 20 samples. For wheat, RBSDV was detected by RT-dPCR in 19 out of 20 samples, compared to 17 out of 20 samples by RT-qPCR (Table 2). These findings suggest that RT-dPCR surpasses RT-qPCR in detecting RBSDV within cereal crop samples, offering enhanced sensitivity and high specificity.

RBSDV can cause significant yield reductions in cereal crops such as wheat, barley, and maize. Yield losses may range from 20% to as high as 80% depending on factors such as the severity of infection, the stage of crop development at the time of infection, and environmental conditions. Additionally, RBSDV-infected grains suffer from various quality defects, including reduced weight, smaller size, chalkiness, and altered chemical compositions. More importantly, RBSDV outbreaks can have long-term consequences for agricultural sustainability, soil health, and ecosystem resilience (Wu et al., 2020). As the current high risk of RBSDV infection in cereal crops increases, developing accurate and sensitive detection methods as early monitoring tools for RBSDV is essential for effective disease management, sustainable agriculture, and safeguarding food

Table 2. Detection of RBSDV in field samples by qPCR and dPCR

Barley			Oat			Wheat		
Sample name	qPCR	dPCR	Sample name	qPCR	dPCR	Sample name	qPCR	dPCR
B-1	N/A	0	O-1	N/A	0	W-1	N/A	0.095
B-2	N/A	0.096	O-2	1.131	0.239	W-2	0.778	0.199
B-3	N/A	0	O-3	N/A	0.048	W-3	0.719	0.102
B-4	N/A	0.239	O-4	1.689	0.575	W-4	N/A	0.05
B-5	N/A	0.048	O-5	1.046	0.096	W-5	N/A	0
B-6	N/A	0.575	O-6	N/A	0.335	W-6	23.7	10.1
B-7	N/A	0.096	O-7	N/A	0.048	W-7	10.4	3.8
B-8	N/A	0.335	O-8	N/A	0	W-8	9.317	5.2
B-9	N/A	0.048	O-9	N/A	0	W-9	0.865	21
B-10	N/A	0	O-10	N/A	0	W-10	0.8	0.356
B-11	N/A	0	O-11	N/A	0.048	W-11	0.6	0.857
B-12	N/A	0	O-12	N/A	0	W-12	201.6	221.8
B-13	N/A	0.048	O-13	N/A	0.191	W-13	34.2	135.3
B-14	N/A	0	O-14	N/A	0.049	W-14	47.3	12.9
B-15	N/A	0.191	O-15	N/A	0	W-15	48.3	24.2
B-16	N/A	0.049	O-16	40.2	35.9	W-16	43.9	21
B-17	N/A	0	O-17	1.478	2.5	W-17	1.6	9.4
B-18	N/A	35.9	O-18	2.985	9.4	W-18	1.8	2.2
B-19	N/A	2.5	O-19	N/A	0	W-19	2.8	2.5
B-20	N/A	9.4	O-20	N/A	0.048	W-20	2.2	1.8
						PC	18,705.1	10,683.8
						NTC	0	0

RBSDV, rice black-streaked dwarf virus; qPCR, quantitative PCR; dPCR, digital PCR; N/A, not available; PC, positive control; NTC, non-template control.

security. By employing diagnostic-based approaches, the spread of the disease to healthy plants can be prevented, and prompt and effective implementation of control measures becomes possible.

Unlike conventional PCR methods, dPCR provides absolute quantification of target nucleic acids without requiring external standards, thus allowing the viral load in samples to be precisely determined. The partitioning of the dPCR reaction into thousands of individual reactions in dPCR improves the quantification precision and accuracy compared to traditional PCR methods (Lei et al., 2021). Given its successful application, dPCR has effectively detected various viral pathogens in crops (Kim et al., 2022b, 2023; Lee et al., 2021).

In this study, we developed an RT-dPCR assay for the detection and absolute quantification of RBSDV in cereal crop leaves without using standard curves. The optimized RT-dPCR revealed high specificity for RBSDV, showing no cross-reactivity with other major cereal viruses found in Korea, including BYDV, BaMMV, and BaYMV. Furthermore, the sensitivity of the RT-dPCR assay for quantifying RBSDV was assessed by testing serial dilutions of RBSDV transcripts, demonstrating its potential applicability across samples with varying virus concentrations. Notably, the sensitivity of RT-dPCR was approximately 10 times greater than RT-qPCR for RBSDV quantification. To verify the reliability and sensitivity of the developed method, 60 cereal crop samples were assessed for both RT-qPCR and RT-dPCR. These findings revealed that RT-dPCR exhibited a greater detection rate for RBSDV compared to RT-qPCR. Therefore, this assay can potentially revolutionize surveillance and management strategies in cereal crops by enabling early detection and precise quantification of the virus in infected plants. Moreover, it could enhance our un-

derstanding of the epidemiology, transmission dynamics, and genetic variability of RBSDV for cereal crop production.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

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