



## Article

# Quantitative Distribution and Transmission of Tea Plant Necrotic Ring Blotch Virus in *Camellia sinensis*

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**Abstract:** Tea plant necrotic ring blotch virus (TPNRBV), which carries four positive-sense single-stranded RNA segments, causes discoloration spots and multiple necrotic ring blotches in tea trees. To understand the distribution and transmission of TPNRBV in tea trees and prevent its spread, a SYBR Green real-time quantitative polymerase chain reaction (RT-qPCR) method for detecting the four virus segments was developed. The limit of detection of RT-qPCR was 3.81, 4.73, 3.58, and 4.64 copies/ $\mu$ L for the four strands of TPNRBV, which was 100-fold more sensitive than conventional PCR for RNA1 detection, 10-fold for RNA2 and RNA3, and 1000-fold for RNA4 detection. Visual observation and RT-qPCR of different tea plant tissues showed that symptomatic mature leaves contained the highest TPNRBV load; the concentrations of the four RNAs in other tissues decreased or were undetectable with increasing distances from symptomatic leaves. TPNRBV did not spread efficiently through seeds, cuttings, or mechanical inoculation, but was transmitted to some tea cultivars, particularly light albinistic varieties such as ‘Zhonghuang 1’ and ‘Huangjinya’, under field conditions. Our TPNRBV detection method is useful for determining the distribution and transmission characteristics of TPNRBV and selecting tissues with the highest viral load for early diagnosis, monitoring, and management of the disease.

**Keywords:** tea tree virus; quantitative real-time PCR; virus detection; virus transmission



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## 1. Introduction

The tea tree (*Camellia sinensis* (L.) O. Kuntze) is an economically important perennial woody plant found in many countries in Asia, Africa, and Latin America. Tea, a non-alcoholic beverage, is consumed by more than 3 billion people across 160 countries [1,2]. Traditionally, tea trees are considered as virus-free because they contain rich polyphenols, which confer long-lasting antiviral properties to trees [3–5]. However, multiple viruses have recently been identified in tea trees in a meta-genomic study based on next-generation sequencing [6]. Nevertheless, relevant research on tea virology is limited.

Tea plant necrotic ring blotch virus (TPNRBV) is among the most abundant viruses in tea trees. TPNRBV was identified through ultrastructural observation and genome analysis. Molecular phylogenetic analysis indicated that TPNRBV is closely related to the Blueberry necrotic ring blotch virus (BNRBV). Both viruses are positive-sense single-strand RNA viruses classified under genus *Blunervirus*, family *Kitaviridae* [6,7]. TPNRBV possesses a 15 kb nucleotide genome divided into four single-strand RNA segments [6]. RNA1 possesses a single ORF, which contains methyltransferase, cysteine-protease, and helicase. RNA2 possesses a single ORF, which contains helicase-RNA polymerase. All enzymes encoded by RNA1 and RNA2 are involved in viral replication and transcription. RNA3 possesses four ORFs encoding four putative proteins with unknown functions and

molecular weights of 14, 29, 22, and 22 kDa. RNA4 possesses a single ORF encoding a putative movement protein [6].

Symptoms of viral infection mainly include chlorosis or discoloration of leaves and dwarfing or developmental abnormalities, which can eventually lead to plant death [8,9]. TPNRBV-infected mature leaves at the bottom of tea trees display symptoms of discoloration and multiple necrotic ring blotches. Premature defoliation occurs once the tea trees are infected by TPNRBV [6]. Similar disease symptoms such as necrotic plant tissue or defoliation are often observed in plants with infections caused by BNRBV, Citrus leprosis virus (CiLV-C), and Hibiscus green spot virus (HGSV), which are in the same family as TPNRBV [10–12]. BNRBV does not move systemically in diseased blueberry and is not transmitted through vegetative propagation. Nevertheless, it infects healthy blueberries under field conditions and causes irregularly shaped, reddish-brown to black rings or blotches, and even leads to premature defoliation [10]. CiLV-C, a *Cilevirus*, is putatively transmitted by mites and infects herbaceous hosts via mechanical inoculation. Localized lesions occur on the leaves, twigs, and fruits after infection with this virus [11]. HGSV, a *Higrevirus*, does not move systemically in host plants and is transmitted by mites. Once infected by HGSV, the symptoms of leprosis are typically observed on the bark and leaves. Foliar symptoms are prominent mostly during the coolest months of the year [12]. In contrast, the distribution of TPNRBV in infected tea trees and its transmission characteristics are not well-understood, and effective detection methods for the virus are lacking.

Virus detection methods include enzyme-linked immunosorbent assay (ELISA) [13], western blotting (WB) [14], direct tissue blot immunoassay (DTBIA) [15], reverse transcription loop-mediated isothermal amplification (RT-LAMP) [16], polymerase chain reaction (PCR) [12], and real-time quantitative PCR (RT-qPCR) [17,18]. So far, only PCR methods have been applied for detecting TPNRBV and have shown some inconsistencies in detection, sensitivity, and quantification compared with RT-qPCR [6]. Thus, an improved RT-qPCR method is needed for TPNRBV diagnosis.

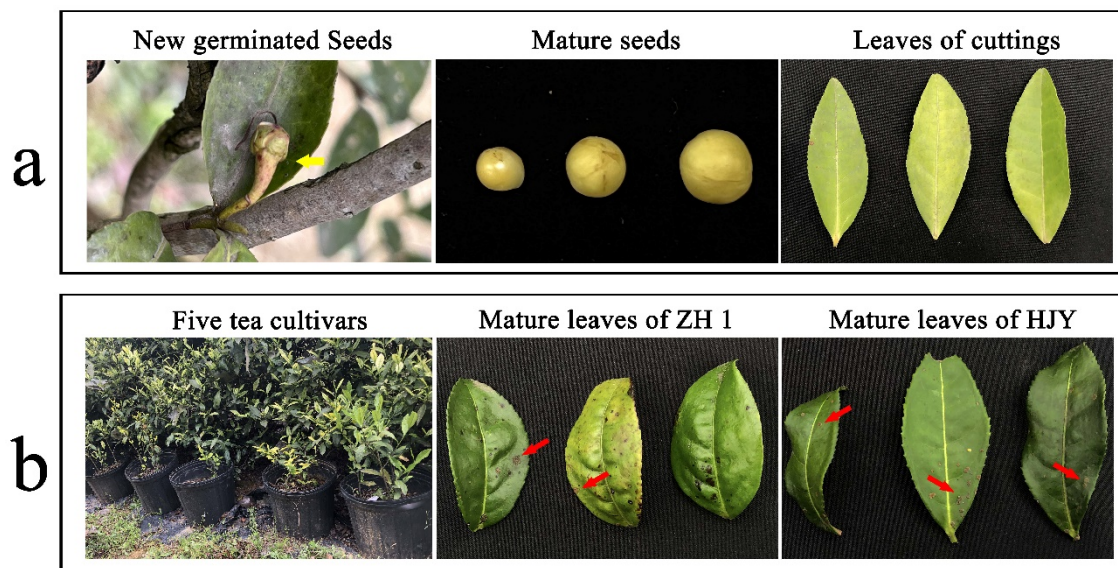
This study aimed to develop a RT-qPCR assay for detection of the four segments of TPNRBV. Furthermore, to better understand the biological importance of TPNRBV, its distribution in diseased tea trees and transmission characteristics were investigated. Data on the quantification, distribution, and transmission of TPNRBV is useful for accurate and effective monitoring and control of TPNRBV.

## 2. Materials and Methods

### 2.1. Plant Materials

The study was conducted in tea gardens at the Tea Research Institute, Chinese Academy of Agricultural Sciences (TRI, CAAS, 30°18'N, 120°10'E), Hangzhou, Zhejiang province, China. Tea cultivar 'Baiye 1' (BY 1), which has a recorded history of TPNRBV infection, was examined. The top buds, first and second leaves of young shoots, new unfolded leaves, asymptomatic mature leaves formed during the current year, asymptomatic mature leaves formed in the previous year, symptomatic diseased mature leaves, green young stems, brown old stems, roots, and flowers were collected for virus detection using RT-qPCR on 16 April, 15 July, and 15 October 2019. Three biological replicates were evaluated in each analysis.

Five groups of seeds (three seeds in each group) formed in spring and five groups of older mature seeds formed in fall (Figure 1a) of TPNRBV-diseased 'BY 1' trees were collected for virus detection. Asymptomatic stems from the middle-upper part of diseased 'BY 1' were planted in tea plant breeding gardens in Fuyang (30°05' N, 119°95' E), Zhejiang province, in 2017. Three groups of mature leaves (three leaves in each group) of cuttings (Figure 1a) were collected for the TPNRBV assay on 14 May 2020.



**Figure 1.** Transmission and infectivity of TPNRBV. (a) Phenotype of seeds and leaves of cuttings derived from TPNRBV-infected ‘BY 1’. (b) Five tea cultivars exposed to TPNRBV-infected ‘BY 1’ gardens for one year. Red arrows indicate necrotic ring blotch symptom.

Two-year-old TPNRBV-free potted tea cuttings from three light albinistic cultivars (‘Zhonghuang 1’ (ZH 1), ‘Zhonghuang 2’ (ZH 2), ‘Huangjinya’ (HJY)), one temperature-sensitive albinistic cultivar (‘Baiye 1’ (BY 1)), and one green cultivar (‘Longjing43’ (LJ 43)) were placed in TPNRBV-positive open tea gardens to assess the transmission of TPNRBV in May 2019 (Figure 1b). After one year, mature and new leaves were sampled for TPNRBV detection. Three biological replicates were evaluated in each analysis.

### 2.2. Virus Supernatant Preparation, Inoculation, and Plant Culture

Symptomatic diseased leaves of ‘BY 1’ were collected from the tea garden. Leaves (100 g), 300 mL of 30 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES) buffer (pH 7.5), and a DFT-200A homogenizer (Wenling Linda Machinery Co., Ltd., Zhejiang, China) were refrigerated at 4 °C for 30 min. The leaves were homogenized in HEPES, and 40 µL concentrated Antifoam A (Sigma, St. Louis, MO, USA) was added. This mixture was centrifuged for 20 min at 1520× *g*, and then the supernatant was centrifuged for 20 min at 11,000× *g* in 50 mL tubes [19]. The supernatant was used as an inoculum to infiltrate the second and third true leaves of two-year-old TPNRBV-free ‘BY 1’ cuttings using needleless syringes. These inoculated tea cuttings were transferred into a growth room under a 12/12 h day/night cycle at 25 °C. Inoculated and adjacent leaves were sampled for the TPNRBV assay 36 days after infiltration.

### 2.3. Total RNA Isolation and Reverse Transcription

All samples were collected and promptly frozen in liquid nitrogen and stored at −80 °C until nucleic acid extractions. RNA was extracted from 100 mg tea tree tissues using an RNAPrep Pure Plant Plus Kit (Tiangen, Beijing, China) according to the manufacturer’s instructions. The quality and concentration of RNA samples were analyzed via 1.0% agarose gel electrophoresis and monitored at 260 and 280 nm using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). First-strand cDNA was synthesized using 1 µg total RNAs in a 20 µL reaction, and the PrimerScript RT reagent kit with gDNA Eraser (Takara Bio Inc., Kusatsu, Japan) was used according to the manufacturer’s protocol. The cDNA products were diluted 10-fold with ultrapure water for subsequent analysis.

#### 2.4. Construction of Recombinant Plasmid Containing cDNA Clones of TPNRBV

cDNA derived from symptomatic diseased leaves of ‘BY 1’ was used as a template for TPNRBV cloning. The genome sequence information on TPNRBV was found under GenBank accession numbers MG781152, MG781153, MG781154, and MG781155 [6]. Because the TPNRBV1 (5.9 kb) sequence is very long, it was obtained through artificial synthesis (Shanghai HuaGen Biotech, Shanghai, China). TPNRBV2 (4.1 kb) was divided into two segments for amplification from the cDNA template using the primer pairs TPNRBV2-F1/TPNRBV2-MR1 and TPNRBV2-MF2/TPNRBV2-R2. TPNRBV3 (2.7 kb), and TPNRBV4 (2.3 kb) was separately amplified from the cDNA template using the primer pairs TPNRBV3-F/TPNRBV3-R and TPNRBV4-F/TPNRBV4-R, respectively. The four full-length cDNA fragments of TPNRBV were individually ligated into the *EcoRI/KpnI*-digested binary expression vector pCAMBIA1300 (9.3 kb) using a GBclonart Seamless cloning kit (Genbank Biosciences, Suzhou, China), resulting in pTPNRBV1, pTPNRBV2, pTPNRBV3, and pTPNRBV4 with sizes of 15.2, 13.4, 12, and 11.4 kb, respectively. These recombinant plasmids of TPNRBV were serially diluted with cDNA from a virus-free sample and used for further PCR and RT-qPCR analyses. The primers used for cDNA cloning and homologous recombination of TPNRBV are listed in Table S1.

#### 2.5. Conventional PCR Analysis

The serially diluted recombinant plasmids of TPNRBV were used for PCR analysis. We previously designed primers used for the detection of four segments of TPNRBV via RT-PCR [6]. Based on the research, three pairs of primers within the four amplicons were redesigned to detect each segment and to select the most specific pairs for PCR and RT-qPCR analysis. The sequence information is shown in Table S2. The 20  $\mu$ L PCR mixture contained 10  $\mu$ L Premix Taq<sup>TM</sup> (TaKaRa Taq<sup>TM</sup> Version 2.0 plus dye) (Takara), 1  $\mu$ L of cDNA template, 1  $\mu$ L of each primer, and 7  $\mu$ L RNA-free ddH<sub>2</sub>O. The PCR program was as follows: 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s. PCR products were evaluated using 1.5% gel electrophoresis.

#### 2.6. SYBR Green Real-Time Fluorescence qPCR Analysis

The serially diluted recombinant plasmids of TPNRBV and different tissues of tea trees were used for RT-qPCR, which was conducted using a Light<sup>®</sup> 480 Real-Time PCR System (Roche, Basel, Switzerland) with 2  $\mu$ L cDNA template, 0.5  $\mu$ L of each primer (10  $\mu$ mol/L), 2  $\mu$ L ultrapure water, and 5  $\mu$ L SYBR Green I Master Mix (Roche). Amplification was performed as previously described [20] as follows: 94 °C for 10 min, 45 cycles at 95 °C for 20 s, 58 °C for 10 s, and 70 °C for 35 s. Primer specificity was evaluated by measuring the melting curve from 55 to 95 °C.

#### 2.7. Quantification of the Four Segments of TPNRBV and Data Analysis

The load of each segment of TPNRBV in different tissues was quantified using a standard curve method. The recombinant plasmids pTPNRBV1, pTPNRBV2, pTPNRBV3, and pTPNRBV4 were serially diluted 10-fold and then used for RT-qPCR analysis with four replicates. The amplification efficiencies (E) were calculated according to the following formula:  $E = 10^{-1/\text{slope}} - 1$ . The plasmid copy number was calculated according to the following formula: numbers of copies = (Avogadro’s number  $6.02 \times 10^{23}$ )  $\times$  (amount of plasmid in nanograms)/(length of DNA in base pairs (bp)  $\times$  660  $\times$  1  $\times 10^9$ ) [18]. The standard curve was prepared with the C<sub>t</sub> value on the vertical axis and log<sub>10</sub> (copy number) on the horizontal axis. To accurately quantify the virus content of all samples, *CsPTB* (Polypyrimidine tract-binding protein; accession number: GAAC01052498.1) and *CsEF1* (Elongation factor; accession number: KA280301.1) [21] of tea plant were used as an internal control and amplified in parallel with four segments of TPNRBV. The mean C<sub>t</sub> values of *CsPTB* and *CsEF1* were 23.0 and 18.9, respectively. Then, the C<sub>t</sub> value of *CsPTB* and *CsEF1* of each sample was divided by 23.0 and 18.9 to obtain the respective ratios. The C<sub>t</sub> value of each sample was divided by mean ratio value of *CsPTB* and *CsEF1* to obtain

the normalized  $C_t$  value, which was substituted into the standard curve to calculate the copy number of each segment of TPNRBV. The primer sequence information for *CsPTB* and *CsEF1* amplification is shown in Table S2. Each sample was detected using RT-qPCR in two technical replicates. The raw  $C_t$  values of all samples are listed in Table S3.

### 3. Results

#### 3.1. Primer Pair Optimization and Establishment of Standard Curve for the Four Segments of TPNRBV

The cDNA clones of each segment were separately constructed into pCAMBIA1300. The recombinant plasmids pTPNRBV1 (5.9 kb), pTPNRBV2 (4.1 kb), pTPNRBV3 (2.7 kb), and pTPNRBV4 (2.3 kb) were verified by gel electrophoresis and sequencing (Figure S1). In addition, three primer pairs were designed to detect each segment, and the most specific pairs were selected for RT-qPCR analysis. To generate the standard curves of the four segments of TPNRBV, a 10-fold dilution series of each plasmid was used for standard curve construction. The four amplicons displayed a single peak in the amplification curves, and a single amplicon appeared on the melting curves (Figures S2 and S3). A linear relationship was observed between  $\log_{10}$  copy number of each plasmid and the  $C_t$  value. The standard curve equation, regression coefficient ( $r^2$ ), and amplification efficiencies ( $E$ ) of pTPNRBV1, pTPNRBV2, pTPNRBV3, and pTPNRBV4 are reported in Figure 2. Thus, the RT-qPCR method was reliable for TPNRBV screening.

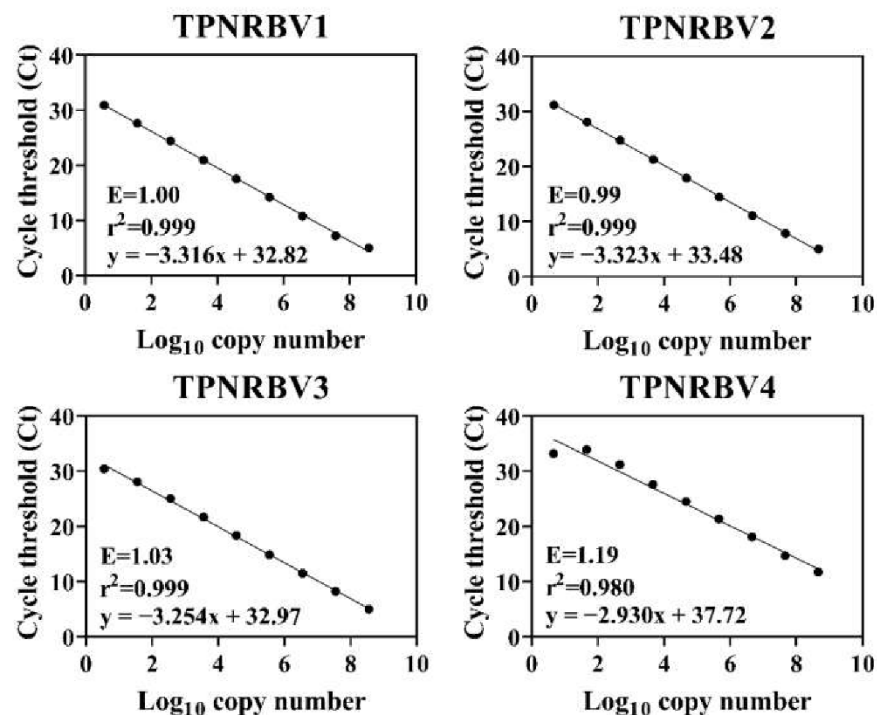
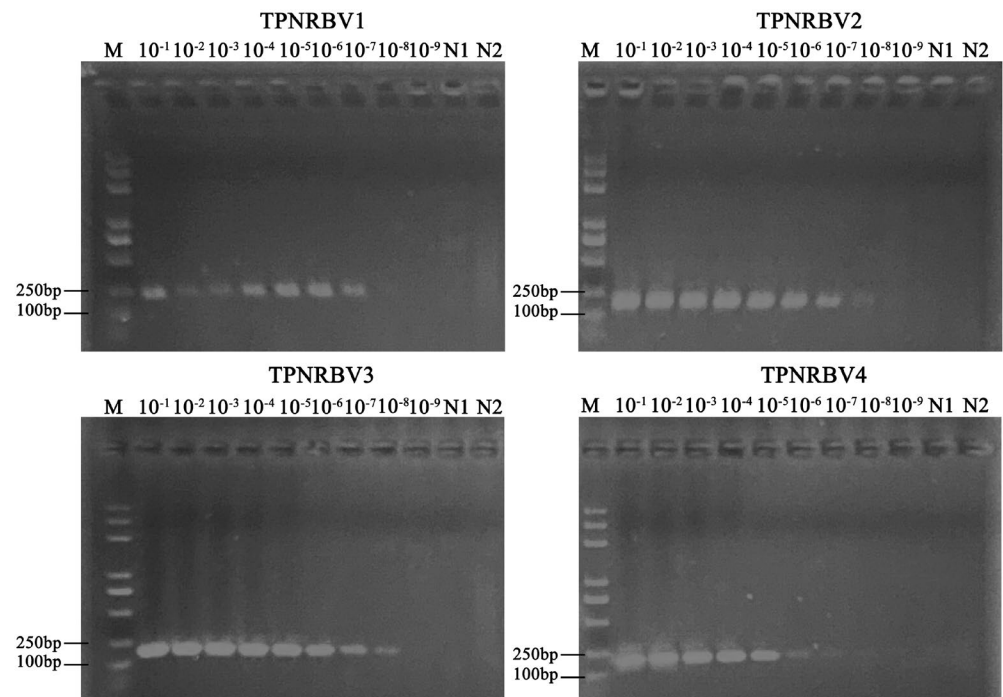


Figure 2. Standard curves of TPNRBV determined using RT-qPCR.

#### 3.2. Sensitivity Comparison of RT-qPCR and PCR

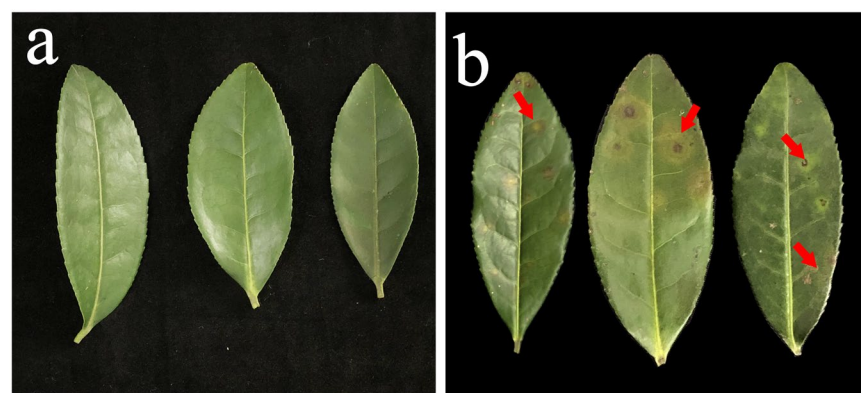
The original liquid concentrations of pTPNRBV1, pTPNRBV2, pTPNRBV3, and pTPNRBV4 were  $3.81 \times 10^9$ ,  $4.73 \times 10^9$ ,  $3.58 \times 10^9$ , and  $4.64 \times 10^9$  copies/ $\mu\text{L}$ , respectively. After serially diluting the plasmids by 10-fold, the standard plasmids were used as template for PCR and RT-qPCR detection. The minimum detectable concentrations of pTPNRBV1, pTPNRBV2, pTPNRBV3, and pTPNRBV4 via PCR were  $3.81 \times 10^2$ ,  $4.73 \times 10^2$ ,  $3.58 \times 10^2$ , and  $4.64 \times 10^3$  copies/ $\mu\text{L}$ , respectively (Figure 3). The detection limits using RT-qPCR were 3.81, 4.73, 3.58, and 4.64 copies/ $\mu\text{L}$ , respectively (Figure S3). These results indicate that the RT-qPCR method is more sensitive than PCR in detecting TPNRBV.



**Figure 3.** Detection of different concentrations of TPNRBV plasmid using PCR. **M**—Marker; **Lanes 2–9**—10-fold serial dilutions of plasmids; **N1**—Negative control using cDNA from TPNRBV-free sample as template; **N2**—Negative control using water as template.

### 3.3. Titer and Distribution of TPNRBV in Diseased Tea Trees within a Year

Phenotypic observation and virus detection were conducted in TPNRBV-diseased ‘BY 1’ to reveal the content and distribution regularity of TPNRBV and its relationship with disease symptoms in tea trees. Necrotic ring blotches were observed on the lower mature leaves of diseased ‘BY 1’ during different seasons, whereas other tissues remained asymptomatic (Figure 4). The virus load was higher in symptomatic mature leaves throughout the year compared to those in other tissues (Table 1). These results indicate that TPNRBV was mainly present and led to diseased symptoms in mature leaves at the bottom of tea bushes. In addition, TPNRBV was detected in both symptomatic and asymptomatic areas of the same diseased leaf, but the virus content of the former was higher than that of the latter.



**Figure 4.** Typical phenotype of TPNRBV infection in ‘Baiye 1’ (BY 1). (a) Asymptomatic mature leaves. (b) Symptomatic diseased mature leaves.

**Table 1.** Detection of TPNRBV in different tissues of diseased ‘Baiye 1’ (BY 1).

Samples	RNA1	RNA2	RNA3	RNA4
	Spring (1 April 2019)			
Top buds	ND, ND, ND	ND, 2.18 × 10, ND	ND, 1.93 × 10, ND	ND, ND, ND
The first upper leaves	ND, ND, ND	ND, ND, ND	ND, ND, ND	2.89 × 10, ND, ND
The second upper leaves	ND, ND, ND	ND, ND, ND	5.67, 7.52, ND	1.78 × 10, 3.34 × 10, ND
New unfolded leaves	-	-	-	-
Asymptomatic mature leaves formed during current year	-	-	-	-
Asymptomatic mature leaves formed previous year	ND, 9.99 × 10 <sup>2</sup> , 1.92 × 10 <sup>3</sup>	8.07, 3.64 × 10 <sup>3</sup> , 5.24 × 10 <sup>3</sup>	ND, 2.10 × 10 <sup>4</sup> , 4.32 × 10 <sup>4</sup>	ND, 1.71 × 10 <sup>6</sup> , 2.62 × 10 <sup>5</sup>
Symptomatic diseased mature leaves	2.13 × 10 <sup>5</sup> , 8.66 × 10 <sup>4</sup> , 2.29 × 10 <sup>5</sup>	3.09 × 10 <sup>5</sup> , 1.36 × 10 <sup>3</sup> , 4.50 × 10 <sup>5</sup>	1.87 × 10 <sup>6</sup> , 3.74 × 10 <sup>5</sup> , 2.76 × 10 <sup>6</sup>	1.19 × 10 <sup>8</sup> , 6.42 × 10 <sup>6</sup> , 4.43 × 10 <sup>7</sup>
Green young stems	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
Brown old stems	2.82 × 10 <sup>2</sup> , 4.75, ND	7.38 × 10 <sup>2</sup> , 2.93 × 10, ND	5.74 × 10 <sup>3</sup> , 7.89 × 10, 1.18 × 10	3.73 × 10 <sup>4</sup> , 2.97 × 10 <sup>2</sup> , 1.63 × 10 <sup>2</sup>
Roots	ND, ND, ND	1.75 × 10, ND, ND	4.11 × 10, 5.80, 1.26 × 10	1.30 × 10 <sup>2</sup> , ND, 8.95 × 10
Flowers	-	-	-	-
Summer (7 July 2019)				
Top buds	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
The first upper leaves	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
The second upper leaves	ND, 1.60 × 10, ND	ND, 2.21 × 10, ND	ND, 2.07 × 10, ND	ND, 3.18 × 10, ND
New unfolded leaves	ND, 1.73 × 10 <sup>4</sup> , ND	ND, 5.02 × 10 <sup>4</sup> , ND	8.26, 1.38 × 10 <sup>5</sup> , ND	1.35 × 10 <sup>2</sup> , 5.37 × 10 <sup>5</sup> , 5.47
Asymptomatic mature leaves formed during current year	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
Asymptomatic mature leaves formed previous year	-	-	-	-
Symptomatic diseased mature leaves	1.24 × 10 <sup>4</sup> , 1.46 × 10 <sup>4</sup> , 1.42 × 10 <sup>4</sup>	1.04 × 10 <sup>4</sup> , 2.69 × 10 <sup>4</sup> , 3.27 × 10 <sup>4</sup>	8.86 × 10 <sup>4</sup> , 2.76 × 10 <sup>5</sup> , 1.25 × 10 <sup>5</sup>	9.76 × 10 <sup>5</sup> , 2.04 × 10 <sup>6</sup> , 2.46 × 10 <sup>6</sup>
Green young stems	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
Brown old stems	ND, 1.3 × 10, ND	ND, 4.08 × 10, ND	7.73, 7.17 × 10, ND	7.94 × 10, 4.36 × 10 <sup>3</sup> , ND
Roots	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
Flowers	-	-	-	-
Autumn (15 October 2019)				
Top buds	ND, ND, ND	ND, ND, ND	2.11 × 10, ND, ND	2.72 × 10, ND, ND
The first upper leaves	ND, ND, ND	ND, ND, ND	2.44 × 10, ND, 7.67	1.80 × 10 <sup>2</sup> , ND, ND
The second upper leaves	ND, 1.51 × 10, ND	ND, 1.50 × 10, ND	5.16 × 10, 9.27 × 10, 1.10 × 10	7.90 × 10, 6.08 × 10 <sup>2</sup> , 1.02 × 10
New unfolded leaves	-	-	-	-
Asymptomatic mature leaves formed during current year	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, 7.46
Asymptomatic mature leaves formed previous year	-	-	-	-
Symptomatic diseased mature leaves	3.16 × 10 <sup>4</sup> , 1.37 × 10 <sup>4</sup> , 1.08 × 10 <sup>4</sup>	1.38 × 10 <sup>5</sup> , 8.18 × 10 <sup>4</sup> , 6.64 × 10 <sup>4</sup>	1.61 × 10 <sup>6</sup> , 8.43 × 10 <sup>5</sup> , 8.74 × 10 <sup>5</sup>	4.20 × 10 <sup>7</sup> , 2.83 × 10 <sup>7</sup> , 2.62 × 10 <sup>7</sup>
Green young stems	ND, ND, 2.33 × 10	ND, ND, 2.19 × 10	7.33, 2.23 × 10, 2.62 × 10 <sup>2</sup>	5.33 × 10, 4.12 × 10 <sup>2</sup> , 3.10 × 10 <sup>3</sup>
Brown old stems	ND, 1.93 × 10, ND	ND, 3.22 × 10, ND	4.83 × 10, 5.25, 5.89	3.99 × 10 <sup>2</sup> , 1.28 × 10 <sup>3</sup> , 1.67 × 10 <sup>2</sup>
Roots	ND, ND, ND	ND, ND, 1.32 × 10	5.69, 3.35 × 10, 6.18 × 10	ND, 1.59 × 10 <sup>2</sup> , ND
Flowers	1.10 × 10, ND, ND	ND, ND, 8.46 × 10	8.77 × 10 <sup>2</sup> , 1.01 × 10, 5.17	9.92 × 10 <sup>3</sup> , 7.12 × 10, ND
Winter (27 December 2019)				
Symptomatic area of diseased mature leaves	7.66 × 10 <sup>4</sup> , 9.05 × 10 <sup>4</sup> , 9.92 × 10 <sup>3</sup>	5.62 × 10 <sup>4</sup> , 6.66 × 10 <sup>4</sup> , 2.02 × 10 <sup>4</sup>	1.14 × 10 <sup>6</sup> , 9.16 × 10 <sup>5</sup> , 5.29 × 10 <sup>5</sup>	2.62 × 10 <sup>7</sup> , 2.49 × 10 <sup>7</sup> , 5.88 × 10 <sup>6</sup>
Asymptomatic area of diseased mature leaves	1.76 × 10 <sup>2</sup> , 2.86 × 10 <sup>2</sup> , 7.63 × 10 <sup>3</sup>	4.59 × 10 <sup>2</sup> , 7.63 × 10 <sup>2</sup> , 5.15 × 10 <sup>3</sup>	7.59 × 10 <sup>2</sup> , 8.10 × 10 <sup>2</sup> , 9.66 × 10 <sup>4</sup>	7.07 × 10 <sup>4</sup> , 6.83 × 10 <sup>4</sup> , 2.38 × 10 <sup>6</sup>

Note: The ‘-’ symbol indicates that these samples were not available. ND indicates that the virus was not detected. Three biological and two technical replicates were evaluated in each sample. The numbers in the table represent copy number of TPNRBV.

With the increasing distance from symptomatic leaves, the copy number of TPNRBV in other tissues gradually decreased or was not detectable (Table 1). Asymptomatic mature leaves formed in the previous year showed a higher virus load than that of newly mature leaves formed during the current year. In addition, these new unfolded leaves transitioning from albino to green showed a higher virus load than that of asymptomatic mature leaves formed during the current year (Table 1).

In spring, the copy numbers of the four segments were larger in the symptomatic mature leaves and brown old stems than those in summer and autumn (Table 1). Thus, spring was more conducive for the replication of TPNRBV.

### 3.4. Transmission and Infectivity of TPNRBV through Tea Seeds and Cuttings, and Mechanical Inoculation under Natural Conditions

RT-qPCR analysis of the transmission of TPNRBV through progeny showed that newly germinated seeds and older mature seeds from TPNRBV-infected ‘BY 1’ were TPNRBV-negative. In addition, leaves of two-year-old cuttings from TPNRBV-infected ‘BY 1’ tea trees were asymptomatic and TPNRBV-negative (Figure 1a). These results suggest that TPNRBV cannot be transmitted through seeds and cuttings.

To investigate the infectivity of TPNRBV via mechanical inoculation, sap (the concentrations of the four segments of TPNRBV were  $5.32 \times 10^4$ ,  $8.82 \times 10^4$ ,  $7.79 \times 10^5$ , and  $4.34 \times 10^7$  copies/ $\mu\text{L}$ , respectively) from symptomatic TPNRBV-positive mature leaves was inoculated into TPNRBV-free tea cuttings of ‘BY 1’. After 36 days, the four RNA segments were not detected via RT-qPCR in inoculated leaves and adjacent non-inoculated leaves, and these leaves did not express disease symptoms. Leaves of non-inoculated control tea trees were TPNRBV-negative and asymptomatic.

To determine the infectivity of TPNRBV under natural conditions, potted cuttings of five TPNRBV-free tea cultivars were placed in TPNRBV-diseased tea gardens. After one year, upper new leaves of all five tea cultivars remained asymptomatic. However, various degrees of shrinkage and mottled blotches appeared on the lower mature leaves of the light albinistic tea cultivars ‘ZH 1’ and ‘HJY’. All four RNA segments were present in the mature leaves of the two cultivars, and no RNA segments were detected in the upper new leaves. In addition, the lower mature leaves of ‘ZH 2’ were asymptomatic, although the four RNA segments were detected in some mature leaves. The mature and new leaves of the other two tea cultivars, ‘BY 1’ and ‘LJ 43’, were asymptomatic and TPNRBV-negative (Figure 1b; Table 2). These results suggest that light albinistic tea cultivars are more sensitive than other cultivars to TPNRBV under field conditions.

**Table 2.** Detection of TPNRBV in five tea cultivars exposed to TPNRBV-diseased ‘BY 1’ gardens for 1 year.

Tea Cultivars	Different Parts of Tea Leaves	TPNRBV			
		RNA1	RNA2	RNA3	RNA4
ZH 1	Upper new leaves	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
	Lower mature leaves	$1.92 \times 10^4$ , ND, $3.18 \times 10^3$	$9.03 \times 10^3$ , ND, $7.76 \times 10^2$	$1.54 \times 10^5$ , ND, $2.03 \times 10^4$	$1.04 \times 10^6$ , ND, $9.81 \times 10^4$
ZH 2	Upper new leaves	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
	Lower mature leaves	ND, ND, $9.03 \times 10^2$	ND, ND, $2.42 \times 10^2$	6.02, ND, $6.11 \times 10^3$	ND, ND, $1.80 \times 10^4$
HJY	Upper new leaves	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
	Lower mature leaves	ND, ND, $8.28 \times 10$	ND, ND, $6.02 \times 10$	ND, ND, $9.03 \times 10^2$	ND, ND, $5.61 \times 10^2$
BY 1	Upper new leaves	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
	Lower mature leaves	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
LJ 43	Upper new leaves	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
	Lower mature leaves	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
Mature leaves of the five tea cultivars before treatment	ZH 1	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
	ZH 2	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
	HJY	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
	BY 1	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND
	LJ 43	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND

Note: ND indicates that the virus was not detected. Three biological and two technical replicates were evaluated in each sample. The numbers in the table represent copy number of TPNRBV.



#### 4. Discussion

According to the classification of the International Committee on Taxonomy of Viruses, TPNRBV is categorized in the genus *Blunervirus*, family *Kitaviridae*, which also contains the genera *Cilevirus* and *Higrevirus*. Viruses in the family *Kitaviridae* contain multipartite segments [7]. Typically, only partial RNAs are used to detect viruses using PCR. RNA4 is used for BNRBV detection, movement protein gene for CiLV-C, and RNA1 for HGSV [10,12,22]. However, monitoring of partial RNA alone is insufficient for establishing the existence and content of each RNA strand in plants. A PCR method was previously established for TPNRBV detection, and multiple tissues were found to be TPNRBV-positive in tea trees [6]. But the limited quantity of samples and low sensitivity of the PCR method may not be sufficient to accurately determine the characteristics of TPNRBV, including its mobility. In this study, the primer pairs were optimized, and a RT-qPCR method which is more sensitive than PCR for TPNRBV detection was developed. To accurately calculate the virus content, all the  $C_t$  values for the detection of viral segments were adjusted based on the coefficient of difference from the average of reference  $C_t$  values. The calculative strategy in this study might provide a useful exploration for accurate calculation of virus content based on internal reference. Disease symptoms mainly appeared on mature leaves at the bottom of tea bushes; the four RNAs showed the highest loads in these leaves. Other tissues showed no disease symptoms, regardless of the presence of the four RNAs. Generally, phenolic compounds in tea trees exert antiviral properties [23]. With increasing leaf maturity, the polyphenol content and vitality decrease [24–26]. In addition, mature leaves in the lower portion of tea bush have a longer growth period, which may give the virus more time for proliferation and accumulation. Thus, the low concentration of polyphenols, inferior vitality, and long growth period of mature leaves may account for these observations. Furthermore, early diagnosis of TPNRBV may be possible through phenotype monitoring and sequence detection of mature leaves in the lower portion of tea trees.

Most viruses in the family *Kitaviridae* lack systemic mobility [27]. BNRBV lacks systemic mobility and does not move from one half of the symptomatic leaf to the other half in an asymptomatic part of the leaf [10]. CiLV can move along mid vein or secondary veinlets in host plants but does not show systemic mobility [11]. HGSV lacks systemic mobility in host plants, and all detected asymptomatic leaves were HGSV-negative [12]. TPNRBV was detected in both symptomatic and asymptomatic areas of the same diseased leaf, and was also present in some remote asymptomatic tissues. It is speculated that TPNRBV might break through the binding of veins and tissues. Compared with BNRBV and HGSV, TPNRBV showed stronger mobility over a short distance. Notably, in symptomatic mature leaves, the four segments of TPNRBV were detected, but in some asymptomatic tissues, only parts of segments of TPNRBV were detected. Other proteins may be required for TPNRBV movement or spread. Thus, how TPNRBV moves in diseased plants requires further analysis.

Viruses can spread through vertical transmission via progeny and horizontal transmission via different individuals. Tea is mainly propagated through seeds and vegetative clonal propagation. Among vegetative propagation, nodal cuttings are widely used to produce large numbers of homogeneous plants [28,29]. Therefore, determining the transmission pattern is important for controlling TPNRBV. TPNRBV was not transmitted by seeds and cuttings. The limited mobility of TPNRBV in diseased tea trees may restrict the transmission of TPNRBV via progeny. Thus, propagation by seeds and cuttings from TPNRBV-free branches has a low risk of being involved in virus transmission.

TPNRBV was not efficiently transmitted to TPNRBV-free tea trees via inoculation. However, TPNRBV was transmitted to some tea cultivars, particularly light albinistic cultivars such as 'ZH 1' and 'HJY', under natural conditions. Disease symptoms such as necrotic ring blotches were observed, and the four segments of TPNRBV were detected. Therefore, virus control of these cultivars with leaf color variation should be considered. Moreover, phytophagous pests, including mites, aphids, leafhoppers, and whiteflies, are the

main vectors that transmit viruses [30]. CiLV-C and HGSV, which belong to the same family as TPNRBV, are transmitted by mites [11,22,31]. Aphids, leafhoppers, mites, and some other phytophagous arthropods are the main pests of tea trees [32–34]. These organisms may play vital roles in the movement and transmission of TPNRBV. Comprehensive studies are necessary to collect pests in different seasons for TPNRBV analysis to determine the potential transmission vector.

## 5. Conclusions

An accurate and effective RT-qPCR method was developed for TPNRBV detection. Symptom observation and sequence monitoring indicated that necrotic ring blotches caused by TPNRBV were present on the leaves at the bottom of the diseased tea trees, and these symptoms were accompanied by the highest load of TPNRBV. With an increasing distance from the symptomatic mature leaves, the concentration of the four RNAs in other tissues decreased. In addition, TPNRBV transmission was not successful mechanically or via seeds. However, light albinistic tea cultivars were more susceptible to TPNRBV under natural conditions. The efficient RT-qPCR method can be used for TPNRBV detection. This study improves the understanding of the distribution and transmission of the virus.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/f13081306/s1>. Figure S1: Schematic diagram of agarose gel electrophoresis of the inserted sequences of TPNRBV; Figure S2: Melting curves of TPNRBV plasmid by RT-qPCR; Figure S3: Amplification curves of TPNRBV plasmid by RT-qPCR; Table S1: Primers used for the construction of recombinant plasmids containing cDNA clones of TPNRBV; Table S2: RT-qPCR primer sequences; Table S3: Raw  $C_t$  values of all samples.

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