

Establishment of Microfluidic Chip Method for Rapid Screening of *Tobacco Rattle Virus*

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Abstract: *Tobacco rattle virus* (TRV), member of the genus *Tobravirus*, is an important plant pathogen with a wide host range beyond any known plant viruses. It is able to be transmitted by nematodes of the genera *Trichodorus* and *Paratrichodorus* (Trichodoridae). In this paper, a rapid screening method was established for inspection of TRV in seeds or other plant materials, combining microfluidic chip technique and fluorescence detection system. The particular region (located at 496-995) of TRV strain (accession number: KP100069.1) was adopted for primer design, method establishment and improvement. Through analysis, this method was proved to be specific for only detecting sample infected by TRV, while 7 other leaf materials infected by *Tomato spot wilt virus* (TSWV), *Tobacco ringspot virus* (TRSV), *Cucumber mosaic virus* (CMV), *Lily symptomless virus* (LSV), *Lily mosaic virus* (LMV), *Tomato ringspot virus* (ToRSV), and *Prunus necrotic ringspot virus* (PNRSV), respectively, showed negative results. Sensitivity tests represented a detection limit as low as 1.00×10^2 copies/ μ L which is beyond normal PCR assays. In one word, microfluidic chip based analyzing platforms hold high promises to enable high-throughput and high-precision screening with less sample consumption, fast detection, simple operation, multi-functional integration, small size, multiplex detection and portability, thus promoting the development of biosecurity diagnostics.

Keywords: *Tobacco rattle virus* (TRV), Biosecurity, Diagnostic Technology, Microfluidic Chip

1. Introduction

Tobacco rattle virus (TRV), belonging to the genus *Tobravirus* and family *Virgaviridae*, is an important plant pathogen of vegetables, ornamentals, and weed hosts. It has a wide host range beyond any known plant viruses, including those from over 50 monocotyledonous and dicotyledonous plant families [1]. TRV triggers diverse symptoms in many economically important crops, ornamentals and other wild species, such as spraing and stem mottle in potato [2], rattle in tobacco, yellow blotch in sugar beet [3], streaky mottle in narcissus [4] and tulip [5], notched leaf in gladiolus [6] and malaria in hyacinth [7].

Tobraviruses feature a bipartite genome, a “30K”-like cell-to-cell movement protein and transmission vectors of nematodes. Virions of TRV are tubular particles with a central canal 4-5 nm in diameter, able to be transmitted by

nematodes of the genera *Trichodorus* and *Paratrichodorus* (Trichodoridae). Ingested virions get attached to the esophageal wall of either adult or juvenile nematodes, and are supposed to be exported via salivary gland secretions and transmitted into injured root cells during exploratory feeding probes. Virus particles can remain alive for several months in non-feeding nematodes [8]. Seed transmission is also possible for TRV in several host species. TRV particle consists of two positive sensed single stranded RNAs, designated as RNA1 and RNA2. RNA1 is the longer strand with replication capability while the other contains coat protein genes necessary for particle integrity and ORFs required for transmission via nematodes. Isolates with only RNA1 is referred to as NM-type which can cause severe symptoms, yet have limited natural transmission rate by nematodes or experimental transmission capability via leaf sap. Strains with both RNAs are regarded as M-type [9].

Since one-third of plant viruses are seed transmitted, this

has led to significant economic consequences, especially in an era of trade globalization [10]. In this paper, a rapid screening method was established for inspection of TRV in seeds or other plant materials, combining microfluidic chip assay and fluorescence detection system. This method turned out to be sensitive, specific, cost-effective and efficient, which has the potential for further use by quarantine service provider.

2. Materials & Methods

2.1. Sample Preparation

Eight leaf samples (Table 1) infected by TRV, *Tomato spot wilt virus* (TSWV), *Tobacco ringspot virus* (TRSV), *Cucumber mosaic virus* (CMV), *Lily symptomless virus* (LSV), *Lily mosaic virus* (LMV), *Tomato ringspot virus* (ToRSV), and *Prunus necrotic ringspot virus* (PNRSV), respectively, were employed in specificity test. Depending on the particular region of TRV genome from GenBank (accession number: KP100069.1) [11], sequence located at 496-995 was chosen, synthesized and incorporated into plasmid puc57 vector [12] as the positive control for further use (carried out by NingBo iGene Technology Co., Ltd, China).

2.2. RNA Extraction & cDNA Synthesis

Total RNA extraction from 30-50 mg ground leaf tissue of each sample was conducted by using RNeasy Plant Mini Kit 50 (QIAGEN, Germany) according to the introduction. Corresponding purity and concentration of RNA solutions (in RNase free ddH₂O) were checked via Nanodrop 2000 (Thermo, USA), which was followed by cDNA synthesis via TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TRANS, Beijing). Those cDNA solutions were stored at -20°C for further use.

2.3. Primer Design

The particular region (located at 496-995) of TRV strain (accession number: KP100069.1) was adopted for primer design and further method establishment. Web service Primer Explorer v4.0 (<http://primerexplorer.jp/elamp4.0.0/index.html>) was used accordingly [13]. Primer synthesis and purification were undertaken by BGI Co., Ltd (Guangdong).

2.4. Establishment of Microfluidic Chip Assay

Commercial buffer used in this paper was Fluorescent isothermal amplification premix (Ningbo iGene Technology Co., Ltd, China). The reaction volume of 25 µL (for 4 reactions, 5 µL per reaction, the rest for error) contained 8.9 µL premix, 1.1 µL primers, and 15.0 µL template. Microfluidic fluorescence detector MA2000 (Ningbo iGene Technology Co., Ltd, China) was employed for heating the reaction to 63.5°C for 30-60 min. The initial primer concentration was prepared at 100 µM. Eight primer combinations were tested at the fixed volume ratio of F3/B3:FIP/BIP=1:8. The final F3-B3

and FIP-BIP primer pairs were decided based on experimental Ct, according to which LP primer was then designed.

Another analysis depending on two assemblies of primers (F3/B3:FIP/BIP:LP=1:8:4 vs. F3/B3:FIP/BIP:LP=1:7:2, volume ratio) were carried out in order to obtain a higher sensitivity.

2.5. Specificity and Sensitivity Tests

The recombinant puc57 plasmid was prepared at the concentration of 1.00×10^6 copies/µL functioning as parent solution which was serially diluted at a 10-fold gradient with RNase free ddH₂O. This step resulted in 7 solutions ranging from 1.00×10^6 to 1.00×10^0 copies/µL which would be used in sensitivity analysis. Moreover, plasmid solution at 1.00×10^4 copies/µL was also employed in primer selection tests. Meanwhile method specificity was analyzed via 8 leaf materials infected by TRV, TSWV, TRSV, CMV, LSV, LMV, ToRSV and PNRSV (Table 1), respectively. RNase free ddH₂O kept serving as the negative control.

3. Result & Analysis

3.1. Primer Design and Selection

Eight primer sets (F3-B3 and FIP-BIP) were created with the online service Primer Explorer v4.0, namely A2, A6, B2, B6, C2, C6, D2, D6 (Table 2). All the primer sets were subject to microfluidic assays with recombinant puc57 plasmid at the concentration of 1.00×10^4 copies/µL without LP primer. The volume ratio of F3/B3 vs. FIP/BIP was 1:8. Reactions were heated to 63.5°C for 60 min. According to the results, eight reactions were completed in 30 min. Thus we shorten the duration for microfluidic assay to 30 min. Based on corresponding Ct (Table 3), primer set B2 had the lowest one (16.32), so it was selected and related LP primer was designed.

3.2. Primer Volume Ratio Optimization

Depending on experiences, primer volume ratio of F3/B3 vs. FIP/BIP vs. LP was analyzed at two levels, that is, 1:8:4 and 1:7:2. Recombinant plasmid at the concentration of 1.00×10^4 copies/µL was also used here and each treatment had four repetitions. It turned out that Ct of ratio 1:7:2 was lower than that of ratio 1:8:4 (Table 4). Then primer volume ratio in this microfluidic assay was settled at 1:7:2.

3.3. Sensitivity Test

Seven solutions of recombinant puc57 plasmid at the concentration ranging from 1.00×10^6 to 1.00×10^0 copies/µL were used in sensitivity test. Each treatment had three repetitions. Corresponding Ct (Table 5) and amplification curves (Figure 1) showed that the detection limit of this microfluidic assay for TRV was 1.00×10^2 copies/µL.

3.4. Specificity Analysis

In order to confirm the specificity of this microfluidic assay, cDNA samples from 8 leaf materials infected by TRV, TSWV,

TRSV, CMV, LSV, LMV, ToRSV and PNRSV, respectively, were employed. Concentrations of those cDNA samples were adjusted near 5.00×10^5 copies/ μL . Recombinant puc57 plasmid solution (1.00×10^4 copies/ μL) and RNase free ddH₂O

were adopted as positive and negative controls. Both Ct and amplification curves demonstrated that only TRV samples and positive control had typical curves as well as Ct. Other cDNA samples had negative results (Tables 6&7, Figures 2&3).

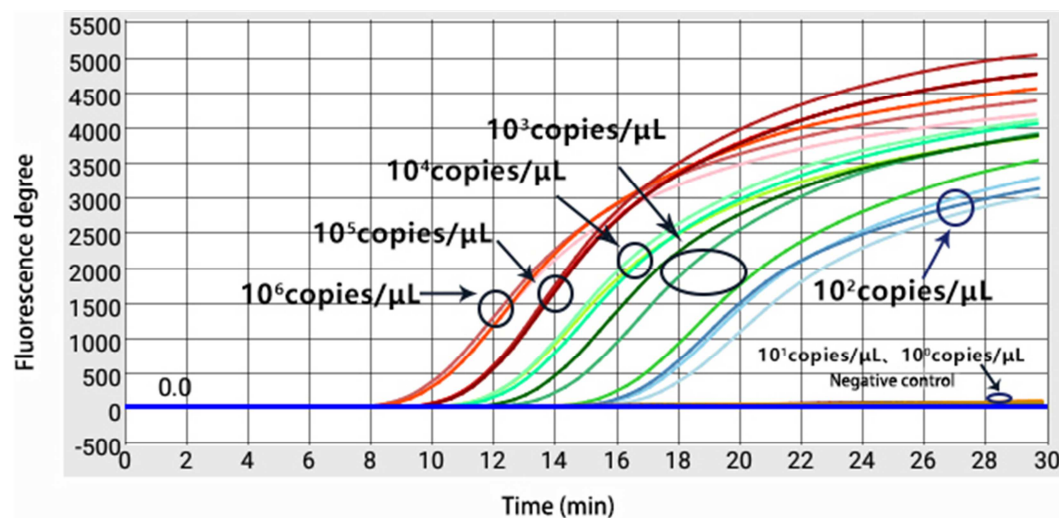


Figure 1. Result of sensitivity test. Blue straight line (0.0) stands for the threshold line.

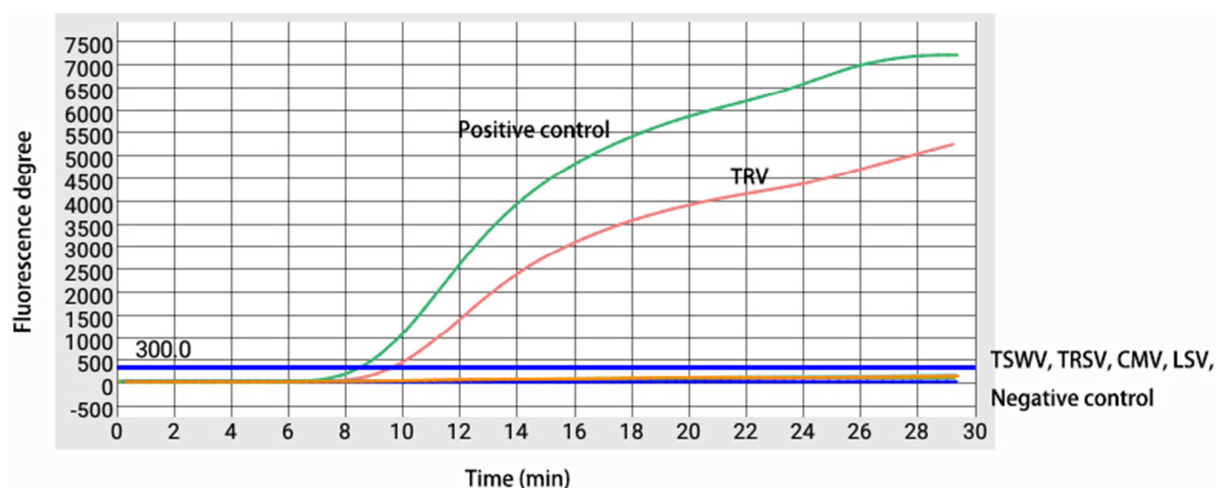


Figure 2. Result of specificity analysis. Blue straight line (300.0) stands for the threshold line.

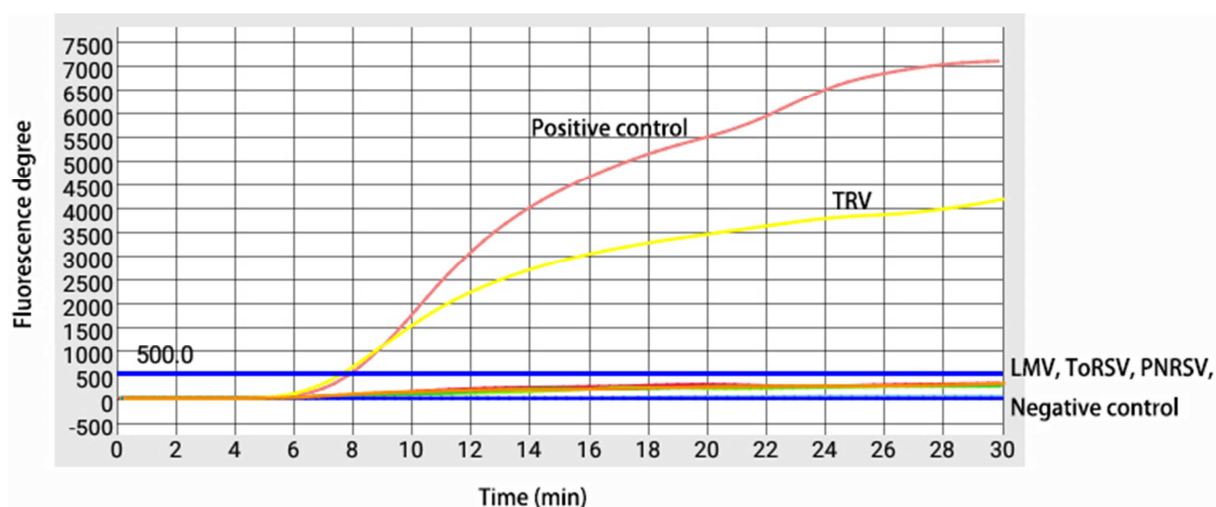


Figure 3. Result of specificity analysis. Blue straight line (500.0) stands for the threshold line.

Table 1. Plant samples used in specificity test.

No.	Samples	Sources
1	Leaves infected by TRV	Reserved sample
2	Leaves infected by TSWV	Agdia Inc. USA
3	Leaves infected by TRSV	Agdia Inc. USA
4	Leaves infected by CMV	Reserved sample
5	Leaves infected by LSV	Reserved sample
6	Leaves infected by LMV	Reserved sample
7	Leaves infected by ToRSV	Agdia Inc. USA
8	Leaves infected by PNRSV	Agdia Inc. USA

Table 2. Eight primer sets created by online service.

No.	Primer	Sequence (5'-3')
A2	F3	ATTCGATAAGGCGGGAGG
	B3	GAGTCTAACAAAAAGTAAACCTTTC
	FIP	GCGCAAATTTTACACTATGCAGTTT-CCTGCAGATTTGATGGATG
	BIP	TACAGTCTGGTAGAGATGAGATCAC-GGAAACTGTTCTGTACGGC
A6	F3	GCATAGTGTAATAATTGCGC
	B3	TCTCTCTTGAAGGCAGA
	FIP	GCGAATAAGGACACTGTCTACTGAG-ACAGTCTGGTAGAGATGAGAT
	BIP	ACGAACAGTTTCTGAAAGAAAGG-GCGTTCTGAATAACACCGA
B2	F3	GCTTGAAAGATCTTTTAAAGAAAC
	B3	CGTTCTGAATAACACCGAGA
	FIP	GAGAGTGGTCAGCAAACCGG-CATAGTGTAATAATTGCGCTAC
	BIP	AGTAGACAGTGTCTTATTCGCC-GCGTTATTAGCACGTGAGT
B6	F3	AACAGTTTCTGAAAGAAAGG
	B3	CCTGTGTTTGCCTTCGTAG
	FIP	GGCTCTCTCTTGAAGGCAGCTCACGTGCTAATAACGCTC
	BIP	TGAGAAGAATGCTGTCGCTGGTACTACCTTGATTGTAGTGACC
C2	F3	CTGGTAGAGATGAGATCACC
	B3	ATTCTTCTCATCGGCTCTC
	FIP	CTTTCTTTCAGGAACTGTTCGTAC-GGTTTGCTGACCACTCTC
	BIP	TTACTTTTGTAGACTCACGTGCT-CTCTTGAAGGCAGAAGCG
C6	F3	ACTACTGGTTACTGAATCACTT
	B3	TGAGAGTGGTCAGCAAAC
	FIP	ATCAAATCTGCAGGCCCTCC-CGCTAACTAACATGGGTGA
	BIP	TGGGTTGAGTCTACAGCTTGA-TCTCATCTCTACCAGACTGT
D2	F3	TTACTGAATCACTTACGCTAAC
	B3	CACTGTCTACTGAGAGTGG
	FIP	ATGAGTCATCCATCAAATCTGCA-GTGACATGTACGATGAGCA
	BIP	GGTTGAGTCTACAGCTTGAAG-TCTCATCTCTACCAGACTGT
D6	F3	ATTCGATAAGGCGGGAGG
	B3	AGAGCGTTATTAGCACGT
	FIP	GCGCAAATTTTACACTATGCAGTTT-CAGATTTGATGGATGACTCAT
	BIP	ATCACCGGTTTGCTGACCAC-AAGTAAACCTTTCTTTTCAGGAA

Table 3. Result of primer set selection test.

No.	Concentration of recombinant plasmid (copies/uL)	Ct
A2	1×10 ⁴	22.15
A6	1×10 ⁴	21.24
B2	1×10 ⁴	16.32
B6	1×10 ⁴	18.12
C2	1×10 ⁴	20.86
C6	1×10 ⁴	21.17
D2	1×10 ⁴	20.98
D6	1×10 ⁴	21.04

Table 4. Result of primer volume ratio analysis.

Volume ratio	No.	Ct
1:8:4	1	13.51
	2	13.18
	3	12.60
	4	13.41
1:7:2	1	10.88
	2	11.02
	3	10.94
	4	10.94

Table 5. Result of sensitivity test.

Concentration (copies/uL)	No.	Ct
10^6	1	8.18796
	2	8.14236
	3	8.14286
10^5	1	9.75524
	2	9.71247
	3	9.75124
10^4	1	10.87896
	2	10.94236
	3	10.94286
10^3	1	13.01699
	2	13.14236
	3	12.14286
10^2	1	14.87896
	2	14.81699
	3	14.84286
10^1	1	0
	2	0
	3	0
10^0	1	0
	2	0
	3	0
Negative Control	1	0
	2	0
	3	0

Table 6. Result of specificity analysis.

Sample	Ct
TRV	8.65674
TSWV	0
TRSV	0
CMV	0
LSV	0
Positive control	8.17514
Negative control	0

Table 7. Result of specificity analysis.

Sample	Ct
TRV	7.96332
LMV	0
ToRSV	0
PNRSV	0
Positive control	8.03261
Negative control	0

4. Discussion

As microfluidic technologies realize manipulation of bioparticles at the microscale through actuating fluids, it has provided multitudinous applications and stimulated the development of life sciences [4]. Technology revolution has

advanced microfluidics towards system miniaturization which results in distinct advantages such as high throughput, enhanced sensitivity, improved analytical ability, facile parallelization, reduced reagent volumes as well as vastly reduced instrumental footprints [14]. Furthermore microfluidic based newly developed chip technologies, such as lab-on-a-chip, three-dimensional (3D) cell culture, organs-on-chip and droplet techniques, have all been established in recent decades, which suggests that microfluidic chips combined with various detection techniques can be vastly applied in the high-throughput screening, detection and mechanistic studies [15]. The microfluidic chip based analyzing platforms hold high promises to enable high-throughput and high-precision screening with less sample consumption, fast detection, simple operation, multi-functional integration, small size, multiplex detection and portability [16]. Thus, it is urgently needed to construct effective methods for on-site, fast and accurate plant pathogen screening with reduced equipment cost and low analysis time.

Among current detection and identification methods, molecular ones are the most popular and most efficient. Several molecular assays for qualitation and quantitation of TRV have been established and applied in routine inspection as well as field survey [17-22]. The sensitivity of conventional RT-PCR was sufficient to detect viral RNA in 10 ng total nucleic acid extracted from infected tissue [18]. TaqMan real-time RT-PCR assays were proved to be able to detect as little as 1 fg initial quantity of plasmid DNA, even more sensitive than conventional RT-PCR [17]. Using an improved protocol involving minimalized sample handling, colorimetric loop-mediated isothermal amplification (LAMP), and final verification by lateral-flow dipstick analysis, researchers achieved a detection limit of 15-78 pg, equal to the sensitivity of RT-PCR [23]. While in this study, sensitivity test revealed that fluorescence based microfluidic chip assay can realize a detection limit as low as 1.00×10^2 copies/ μ L, which is the most sensitive so far.

Protecting national biosecurity is a responsibility shared by government, industry, and people. From perspective of plant protection organization or authorized agent, imported plants are supposed to be free of insect pests and show no symptoms of disease to meet inspection requirements. Prior to export, a phytosanitary certificate is needed, presenting that the plants are free of harmful pests and diseases. Both exportation and importation need inspection. Obviously, molecular techniques like PCR are still the backbone of virus diagnostics. Along with PCR/qPCR, microscopic inspections, identification hosts based bioassays and ELISAs are believed to continue functioning in the future, though they are labour-intensive and time consuming. Plant pathology and biosecurity are experiencing a vital shift in methodology with the blooming of 'omics' technology. All in all, the future of biosecurity diagnostics will approach in the direction of 'more to be done with less' [24].

5. Conclusion

Tobacco rattle virus (TRV), member of the genus *Tobravirus*, is an important plant pathogen with a wide host range beyond any known plant viruses. It is able to be transmitted by nematodes of the genera *Trichodorus* and *Paratrichodorus* (Trichodoridae). In this paper, a rapid screening method was established for inspection of TRV in seeds or other plant materials, combining microfluidic chip technique and fluorescence detection system. Through analysis, this method was proved to be specific for only detecting sample infected by TRV, while 7 other leaf materials infected by TSWV, TRSV, CMV, LSV, LMV, ToRSV and PNRSV, respectively, showed negative results. Sensitivity tests represented a detection limit as low as 1.00×10^2 copies/ μ L which is beyond normal PCR assays. Plant pathology and biosecurity are experiencing a vital shift in methodology with the blooming of 'omics' technology. All in all, the future of biosecurity diagnostics will approach in the direction of 'more to be done with less'.

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