

# Identification of viruses infecting cigar and flue-cured tobacco using next generation sequencing and establishment of multiplex RT-PCR

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## Research

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# Abstract

**Backgrounds:** Infection of plant viruses cause extensive damage to plants and reduce crop yield. Early, precise and simultaneous identification of the plant viruses is of great significance on preventing the spread of the viruses as well as reducing losses on agricultural yield.

**Methods:** Identification of plant viruses from symptomatic samples collected from cigar tobacco planting area in Deyang and flue-cured tobacco planting area in Luzhou city of Sichuan Province China was conducted by the deep sequencing of small RNAs (sRNAs) through an Illumina sequencing platform and plant virus specific contigs were generated based on the virus derived siRNA sequences. Additionally, sequence alignment and phylogenetic analysis was performed to determine the species or strains of these viruses. Subsequently, specific primers were designed for simultaneous detection of five RNA viruses infecting tobacco.

**Results:** A total of 27930450, 21537662 and 28194021 clean reads were generated from three pooled samples and a total of 105 contigs that can be mapped to the closest plant viruses with the length range from 34~1720 nt. The results indicated that the major viruses were potato virus Y (PVY), Chilli veinal mottle virus (ChiVMV), tobacco vein banding mosaic virus (TVBMV), tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV). A fast and sensitive multiplex reverse transcription polymerase chain reaction (RT-PCR) assay was developed for the simultaneous detection of the most frequent RNA viruses infecting cigar and flue-cured tobacco in Sichuan.

**Conclusion:** These results provide theoretical basis and convenient methods for rapid detection and control of viruses on cigar and flue-cured tobacco.

## Background

Crop losses due to plant viral infection poses serious threat to food sustenance and is of great concern worldwide [1]. Plant viruses induce symptoms such as mosaic, mottling, yellowing, ring spots, venial necrosis, stunting, shoestring and deformation of the hosts, which may result in significant losses or quality reduction [1, 2]. Synergistic infection by multiple species of viruses often cause more serious symptoms and damages of the plants [3, 4]. Therefore, early, precise and simultaneous identification of the plant viruses is of great significance on preventing the spread of the viruses as well as reducing losses on agricultural yield. The multiplex RT-PCR assay is a widely-used molecular diagnostic technique used for the amplification of multiple target sequences of different pathogens in a single PCR experiment [5, 6].

RNA silencing in plant is one of the major defense systems to combat viruses, in which the exogenous viral double stranded RNA (dsRNA) is perceived and cut by DICER-like (DCL) proteins to produce primary virus-derived small interfering RNAs (vsiRNAs) and amplified by RNA-dependent RNA polymerases (RDRs) to generate secondary vsiRNAs of between 21 to 25 nt [7, 8]. Global investigation of the vsiRNAs

using next-generation sequencing (NGS) approaches are effective on the identification of the reported or novel viruses [9–11].

Plants belong to Solanaceae, especially tobacco (*Nicotiana tabacum*) is a natural host for a large number of plant viruses [12, 13]. In China, more than 25 plant viruses have been identified from tobacco. TMV is the most well investigated plant virus that cause continuous losses to tobacco production [14]. In addition to TMV, various viruses belong to the genus *Potyvirus* including PVY, ChiVMV, TVBMV and tobacco etch virus (TEV) can also independently or synergistically infect tobacco plants and significantly decrease their quality [12, 15, 16]. PVY is a prevalent virus with wide host range that can induce vein necrotic or leaf mosaic symptoms in tobacco based on their strains [17–19]. Infection of TVBMV can cause bands of dark green tissue along the veins as well as small white to brown spots on the leaves [20]. Additionally, ChiVMV induce leaf punctate necrosis with round bright yellow spots and deformation symptoms on tobacco [15, 21, 22].

Sichuan province is one of the main regions for tobacco production in China and is located in the subtropics area, with abundant species of vegetation, plant viruses as well as their vector insects such as aphids and *Bemisia tabaci*. Especially in cigar tobacco growing areas of Deyang city, tobacco is mixed planted with crops such as peppers and garlic, which significantly increases the risks of transmission of different virus species. In this study, we used NGS technique of small RNA sequencing to identify viruses from symptomatic tobacco plants in farm fields of Deyang and Luzhou of Sichuan province. The results indicated that major viruses are PVY, ChiVMV, TVBMV, TMV and CMV. We also performed sequence alignment to reveal the phylogenetic relationships of the viruses. Additionally, we established a multiplex RT-PCR for simultaneous detection of these viruses, which is applicable for the rapid virus identification and control.

## Methods

### Collection and preparation of samples

In the summer of 2019, more than 80 leaf samples with mosaic, mottling, yellowing, stunting or deformation symptoms of *Nicotiana tabacum* variety cigar (cigar tobacco) with distinct viral symptoms were collected from Deyang city in Sichuan Province of China (Longitude: 104°10'3", latitude: 31°7'36"). In addition, the viral disease symptomatic leaves of *Nicotiana tabacum* variety Yunyan85 and Yunyan87 were collected from Luzhou city in Sichuan province in China (Longitude: 105°38'28", latitude: 28°7'33"). The collected cigar tobacco and flue-cured tobacco leaf samples were immediately frozen in liquid nitrogen and preserved at – 80°C for extraction and some were used for plant inoculation and high-throughput sequencing.

### Small RNA library construction, sequencing and bioinformatics analysis

Total RNA were extracted from the collected cigar tobacco and flue-cured tobacco leaf samples using a mirVana™ miRNA Isolation Kit (Cat #. AM1561, Austin TX, US) according to the manufacturers guidelines and subjected to detection using Agilent 2100 Bioanalyzer (Agilent technologies Santa Clara, USA). The high-throughput sequencing was performed on an Illumina HiSeq-2000 sequencing platform (Shanghai Biotechnology Corporation, Shanghai, China). Briefly, for the symptomatic cigar tobacco and flue-cured tobacco leaf samples small RNA libraries, the low quality reads and 5'- and 3'-adaptor contaminants were clipped from raw reads, and then clean reads ranging from 18- to 28-nt in length, which can be mapped to the genome sequences of plant viruses were extracted for analyses. The contigs with the length range from 34 ~ 1720 nt were assembled based on the virus derived siRNA sequences using the scaffolding contig algorithm (CLC Genomics Workbench version:6.0.4) [23] and Cap3 EST splicing software. Sequences of the contigs were used to query the GenBank databases using the Basic Local Alignment Search Tool (BLAST) and mapped to the closest plant viral genome. Final hits with nucleic acid coverage  $\geq 90\%$  and identity  $\geq 90\%$  of the viruses were identified and aligned to determine their positions.

## **Virus amplification and sequencing validation**

Total RNA was extracted from the above preserved leaves using Eastep Super Total RNA Extraction Kit (Promega, Shanghai, China). Total RNA was used as a template for RT-PCR, and five pairs of specific primers were designed based on alignment of the contigs and the genomic sequences of the PVY (no, MN734254.1), ChiVMV (no, KC711055.1), TVBMV (no, EF219408.1) TMV (no, HE818449.1) and CMV (no, NC-001440.1), respectively, from the GeneBank. PVY-5719F/PVY-6263R, ChiVMV-2441F/ChiVMV-3453R, TVBMV-1071F/TVBMV-2425R, TMV-70F/TMV-2061R and CMV-1257F/CMV-1913R were shown in Table 1. The amplified PCR products were purified using a SanPrep Column PCR Product Purification kit (Sangon Biotech, Shanghai, China) and cloned into the pEASY-T1 vector (TransGen Biotech, Beijing, China).

## **Phylogenetic analysis**

The nucleotide sequences of PVY Vpg, ChiVMV P3, TVBMV HC-pro, the MET domain of TMV p126 together with CP of CMV were amplified using PVY-5719F/PVY-6263R, ChiVMV-2441F/ChiVMV-3453R, TVBMV-1071F/TVBMV-2425R, TMV-70F/TMV-2061R and CMV-1257F/CMV-1913R, respectively, and compared with other related virus isolates whose sequences were deposited in GenBank. Homologous genes were searched using BLAST program in NCBI website. Sequences of the same virus species were aligned by Clustal W version 1.6 and the phylogenetic analysis was performed by using MEGA7 software [24] with the maximum likelihood algorithm and statistical test with 1000 replicates to construct phylogenetic tree of those viruses.

## **Multiplex RT-PCR analysis**

At least 20 different strains of each virus originating from different geographical regions were retrieved from the NCBI database and used for multiple sequence alignment. The sequence identity was at least 98% for each virus and conserved regions were chosen for primer design. Due to the frequent occurrence of CMV in tobacco planting field, we also added CMV detection primers in multiplex RT-PCR analysis.

Each primer pair was designed to amplify the respective virus-specific amplicon of a different length. Five pairs of specific primers including PVY-6494F/PVY-7087R, ChiVMV-7987F/ChiVMV-8809R, TVBMV-989F/TVBMV-2222R, TMV-130F/TMV-614R and CMV-265F/CMV-1240R were designed and shown in Table 1. The plasmid of each of the viruses tested, derived from previous studies, were used as positive controls. cDNAs were synthesized by reverse transcription of the total RNAs using HiScript 1st Strand cDNA Synthesis Kit (Vazyme Biotech, Nanjing, China). The cDNAs were used as the templates (cigar tobacco leaves collected from Deyang with various disease symptoms are numbered as 1 to 16 and symptomatic flue-cured tobacco leaves collected from Luzhou are numbered as 17 to 22, respectively). A series of 10-fold dilutions from  $1 \times 10^8$  to  $1 \times 10^2$  copies of plasmid containing respective viral sequences was performed by using the templates to determine the lowest detection limit. PCR amplification were performed in 25  $\mu$ L final volume. Reaction components included 1  $\mu$ L of 10  $\mu$ M forward and reverse primers, 1  $\mu$ L cDNA and 12.5  $\mu$ L of 2 $\times$  TaKaRa Premix (Ex Taq, 1.25 U/25  $\mu$ L; dNTP Mixture, each 0.4 mmol/L; Mg<sup>2+</sup>, 4 mmol/L) (Takara Bio, Dalian, China), and appropriate ddH<sub>2</sub>O. The PCR procedures were as follows: pre-denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at varying temperatures 55°C for 30 s, and extension at 72°C for various durations 80s; and a final extension at 72°C for 10 min. Finally, PCR products were separated and detected by 1.5% agarose gel electrophoresis and a gel imaging system (Bio-Rad, Hercules, CA, USA) was employed to observe the electrophoresis results.

## Results

### Collection of samples with obvious symptoms on Tobacco and cigar tobacco in Sichuan province

The climate in the Sichuan province is warm and humid with a large variety of plant viruses. In 2019, approximately 80 symptomatic leaf samples of cigar tobacco and flue-cured tobacco were collected from farm fields of Deyang and Luzhou city in Sichuan Province in China. The prevalent viral disease symptoms such as mosaic, venial necrosis, mottling, yellowing and necrotic spots were observed on leaves (Fig. 1). These samples were thereafter subjected to the sequencing analysis and virus identification.

### Identification of major viruses by High-throughput small RNA sequencing

The small RNA libraries of two symptomatic cigar tobacco pooled samples from Deyang city and a symptomatic flue-cured tobacco pooled sample were constructed and sequenced on an Illumina HiSeq-2000 platform which generated 28765749, 25093608 and 34935454 raw reads, respectively, and submitted to the Sequence Read Archive database at NCBI with the accession number SRR13499486, SRR13499485 and SRR13838825. Adapters and low quality reads were trimmed to obtain 27930450, 21537662 and 28194021 clean reads and the Q20 value were 99.01%, 98.86% and 99.07%, respectively. The clean reads of each library were assembled into contigs through the homology-dependent approach

for plant virus identification. These contigs were searched against the non-redundant nucleotide sequence entries of the NCBI database by BLASTn. The results indicated that major viruses infecting flue-cured tobacco and cigar tobacco leaves in Deyang and Luzhou area were PVY, ChiVMV, TVBMV, TMV and CMV. Among which, there were 27, 29, 2, 14 and 33 contigs with the length range from 34 ~ 1720 nt that can be mapped to the genome sequences of PVY, ChiVMV, TVBMV, TMV and CMV (Fig. 2, Table S1).

## Phylogenetic analysis

Phylogenetic analysis was performed by MEGA7 based on the partial nucleotide sequences of PVY, ChiVMV, TVBMV, TMV and CMV collected from Deyang and Luzhou city in Sichuan province to investigate the evolutionary development of these viruses. The sequences of PVY-Deyang (MW419411) and PVY-Luzhou (MW419412) were compared to 15 other PVY isolates reported from various districts as well as ChiVMV (JX088636.1) and TVBMV (NC-009994.1) as out groups were subjected to sequence alignment and phylogenetic analysis (Fig. 3A). The nucleotide sequences of PVY-Deyang and PVY-Luzhou show high identity with 99.65%. The sequence alignment also indicates that PVY-Deyang and PVY-Luzhou are most closely related with PVY-pt077-pot-18 (MN734254.1) on *Solanum lycopersicum* from Zhenjiang city of Jiangsu province China with 100.00% and 99.65% sequence identity, respectively. Results of the phylogenetic analysis also show that PVY-Deyang and PVY-Luzhou are distantly related with PVY-AST (JF928460.1) reported from Brazil and PVY-VNP413 (HG810950.1) reported from Viet Nam (Fig. 3A).

The sequences of ChiVMV-Deyang (MW355622) and ChiVMV-Luzhou (MK405594.1) as well as other 13 ChiVMV isolates together with PVY (KJ634023.1) and TVBMV (NC-009994.1) were subjected to sequence alignment and phylogenetic analysis (Fig. 3B). The results show that ChiVMV-Deyang and ChiVMV-Luzhou are most closely related with ChiVMV-Pp4 (KC711056) and Yp-8 (KC7110055) from pepper plants collected from Sichuan province with 99.42% and 99.03% sequence identity, respectively. Sequence of ChiVMV-Deyang also shows high sequence similarity with ChiVMV-Luzhou by 98.74%. While both of the isolates are distantly related with ChiVMV-GD (KU987835.1), Wenchang (GQ981316.1) and Ca (AJ972878.1) isolates from Guangdong, Hainan province in South China and South Korea (Fig. 3B).

The sequences of TVBMV-Deyang (MW419414) and TVBMV-Luzhou (MW419413) were compared with 14 other TVBMV isolates as well as the ChiVMV (JX088636.1) and PVY (KJ634023.1) to construct phylogenetic tree (Fig. 3C). TVBMV-Luzhou is closely related with TVBMV-XDB-04 (MG880245.1) and YN9.1 (KF444434.1) from tobacco with 99.71% and 97.45% sequence identity, respectively. Interestingly, TVBMV-Deyang is most closely related with TVBMV-YN9.1 (KF444434.1) from tobacco with only 94.03% sequence identity and also shows comparatively lower sequence similarity with TVBMV-Luzhou with 93.01% sequence identity. The results of phylogenetic analysis also indicate that TVBMV-Deyang and TVBMV-Luzhou are distantly related with TVBMV SDWF11 (GU904096.1), YND (EF219408.1), JX (JN630471.1) isolates reported from Shandong province of China (Fig. 3C).

Phylogenetic analysis of TMV-Deyang (MW521350) and TMV-Luzhou (MW521351) were compared with other 18 reported TMV isolates together with a tomato mosaic virus (ToMV) (NC-002692.1) and a tobacco mild green mosaic virus (TMGMV) (NC-001566.1) isolate were used as the out groups (Fig. 3D). The results indicate that TMV-Deyang is closely related with Xiongfán-2 (HE818419.1) and Fumeng (HE818416.1) isolates with 99.75% and 98.95% sequence similarity. While TMV-Luzhou clustered with Xuyong (HE818454.1) as well as Tianzhu-1 (HE818444.1) isolate with 99.90% and 99.15% sequence similarity. The sequences identity of TMV-Deyang and TMV-Luzhou is 98.24% and both of the isolates are distantly related with Ohio V (FR878069.1), Harbin-2 (MH595920.1) and SXFQ (JX993906.1) (Fig. 3D).

The sequences of CMV-Deyang (MW698928) were compared to 20 other CMV isolates reported from various district as well as brome mosaic virus (NC-002028.2) and alfalfa mosaic virus (NC-002025.1) from USA as out groups and were subjected to sequence alignment and phylogenetic analysis (Fig. 3F). No CMV isolates were identified from Luzhou in this investigation. The results showed that CMV-Deyang is most closely related with CMV-RX9 (MH119149.1) and CMV-RX2 (MH119146.1) from Glycine max plants collected from China with 98.93% and 98.78% sequence identity, respectively, while distantly related with Ug90-RNA3 (MG021456.2), CMV-HC-56 (FM999062.1) and CMV-Jol186 (JX025994.1) isolates from Uganda, Thailand and Iran (Fig. 3F).

## **Specificity, sensitivity and application of multiplex RT-PCR**

To simultaneously detect these viruses, we designed specific amplification primers for PVY, ChiVMV, TVBMV, TMV as well as CMV and constructed a multiplex RT-PCR detection system. To determine the sensitivity, a series of plasmids including pEASY-PVY, pEASY-ChiVMV, pEASY-TVBMV, pCB-TMV-SN (no. MG516107) and pEASY-CMV were diluted from  $10^8$  copies to  $10^2$  copies, respectively, and amplified by the multiplex RT-PCR using 25  $\mu$ L reaction system. The results indicated that the detection limit of PVY, ChiVMV, TVBMV, TMV and CMV were  $10^4$ ,  $10^4$ ,  $10^4$ ,  $10^4$  and  $10^5$  copies, respectively (Fig. 4A-E).

Furthermore, we mixed these plasmids at equal ratio and diluted from  $10^8$  copies to  $10^2$  copies each, and subjected to multiplex RT-PCR analysis. The results showed that 5 virus specific bands can generally be amplified from the mixed templates from  $10^8$  to  $10^4$  copies each (Fig. 4F), thereby indicating that the developed multiplex RT-PCR was sensitive and specific for simultaneous detection of PVY, ChiVMV, TVBMV, TMV and CMV.

To test the feasibility of the multiplex RT-PCR for simultaneous detection of these viruses in the field. We tested 22 field-collected symptomatic tobacco leaf samples from Deyang and Luzhou tobacco planting areas (Fig. 5A and B). The number of the leaf samples showed in Fig. 5A were consistent with lane number showed in Fig. 5B. The results revealed that detection rate for PVY, ChiVMV, TVBMV, TMV and CMV were approximately 36%, 55%, 45%, 55% and 9%, respectively. Among which, leaf samples (Fig. 5A and B. 1, 6, 7, 11 and 21) were infected by TMV, CMV, ChiVMV, TMV and ChiVMV, respectively. However, the other 17 leaf samples showed synergistic infection by two or more viruses. For example, two samples (Fig. 5A and B. 3 and 8) were synergistically infected by TVBMV and PVY. Leaf samples (Fig. 5A and B.

16, 20 and 22) indicated synergistic infection of ChiVMV and TMV. Additionally, leaf sample number 2 and 5 (TVBMV, ChiVMV and TMV), 9 (TVBMV, PVY and TMV), 10 (ChiVMV, PVY and TMV) and 18 (TVBMV, ChiVMV and PVY) were synergistically infected by three viruses (Fig. 5A and B).

Table 1  
Primer list used for phylogenetic analysis and multiplex RT-PCR

primer name	sequence	amplification size
PVY-5719F	GGGAAAATAAATCCAAAAG	564 bp
PVY-6263R	TTCATGCTCCACTTCCTGTT	
ChiVMV-2441F	GGTGAAGGAATTGAAAGTAT	1032 bp
ChiVMV-3453R	TTGATGACCCACTGGTTCCT	
TVBMV-1071F	TCAGCAGCAGAACAATTCTG	1374 bp
TVBMV-2425R	ACCAACTCTATAATGCTTCA	
TMV-70F	ATGGCATAACACACAGACAGC	1992 bp
TMV-2061R	CTCCGGATGATCTCCAGCAA	
CMV - 1257F	ATGGACAAATCTGAATCAAC	657 bp
CMV-1913R	CAGGGGTGTTCCAGTTTGA	
PVY-6494F	GATGGGCATTGTGGATTACC	613 bp
PVY-7087R	TCTGGAAGCCATGCACTTGC	
ChiVMV-7987F	AATGGCAATGCGGTATTCAC	842 bp
ChiVMV-8809R	CGACGGCCTTCGTCTTAACC	
TVBMV-989F	TGTGCGAGGTCGTGATGGGG	1253 bp
TVBMV-2222R	GATAGCATGCAAGTGCAACC	
TMV-130F	TCCTTGGTCAATGATCTAGC	485 bp
TMV-614R	GTATTGTGACAGACAGCGTC	
CMV-265F	TTCCGAGGTCATCCGGAATC	995 bp
CMV-1240R	CCTTGAAGTGTTCATCCAC	

## Discussion

In China, plant viruses cause extensive economic losses in tobacco production in spite of efforts on different control strategies [25]. In addition to TMV, viruses belong to the potyvirus genus have become

the major viral pathogens infecting tobacco [12, 15]. Sichuan province is one of the main tobacco production area in China [15], where the tobacco plantation is usually surrounded or mixed with other crops, especially pepper plants, which increases the risk of multiple virus synergistic infection. Therefore, it is of great significance to accurately identify the species of plant viruses and establish specific and sensitive detection techniques for virus control.

To clarify the classification and the genetic relationship of the major viruses infecting tobacco plants in Deyang and Luzhou, we amplified partial sequences of three potyviruses including PVY, ChiVMV and TVBMV and subjected to nucleotide BLAST and phylogenetic analysis. The results indicated that PVY-Deyang and PVY-Luzhou showed high sequence identity with each other, and also closely related with the isolates reported from Henan, Shandong, Heilongjiang and Hainan province in tobacco. We also compared amplified sequences of PVY Vpg and the contig sequences generated from RNA-seq with sequences of PVY<sup>N</sup>, PVY<sup>0</sup> as well as PVY<sup>NTN</sup>. These results indicated high sequence similarity with PVY<sup>0</sup> (data not shown), which suggested that the major PVY infecting tobacco plants in Sichuan province possibly be O strain.

Nucleotide BLAST and phylogenetic analysis suggested that TVBMV-Luzhou showed high similarity with TVBMV isolates previously reported from Shandong, Henan or Sichuan province, but comparably distantly related with that collected from Deyang in cigar tobacco. Interestingly, the sequence similarity between TVBMV-Deyang and the closest reported TVBMV isolate was only 94%. It is possible that mutations or recombination have occurred in the evolution process of TVBMV-Deyang, which required more sampling and precise sequencing analysis of TVBMV to verify this possibility in the future study.

BLAST and phylogenetic analysis using the sequences of P3 of ChiVMV-Deyang or ChiVMV-Luzhou indicated that these isolates showed highest similarity with ChiVMV isolates collected from pepper plants in Sichuan province. This results indicated that the ChiVMV infected tobacco in Deyang and Luzhou should be originated from diseased pepper plants, which were in agreement with our previous study [15]. Synergistic infection of plant viruses can induce more severe disease symptoms on plants, which may cause greater losses and increase in difficulty for virus control. In this investigation, synergistic infection of ChiVMV with TVBMV, TMV or PVY can induce severe necrotic spots (Fig. 4A-18; Fig. 4B lane 18), which can be extensively observed in Luzhou tobacco planting area.

In this work, we designed specific amplification primers and established a sensitive and specific multiplex RT-PCR system for simultaneous detection of five major tobacco RNA viruses including PVY, ChiVMV, TVBMV, TMV and CMV, which is well applicable in tobacco growing areas in Sichuan province. Primer design is critical for the establishment of a practical multiplex RT-PCR detection system that requires the optimization of the specificity and sensitivity of the primers, and the distinguishable sizes of amplicon. In a previous study, a multiplex RT-PCR for the detection and classification of different PVY strains from potato including PVY<sup>N</sup>, PVY<sup>0</sup>, PVY<sup>NTN</sup>, PVY<sup>NW</sup> and PVY<sup>NTN-NW</sup> were developed based on different combination of amplicons [26]. However, we used these primers to test our samples, but failed to amplify specific amplicons, which indicated that this reported multiplex RT-PCR analysis system may not be

applicable in the classification of the precise PVY species that infect tobacco. A previous study has established a multiplex RT-PCR analysis for simultaneous detection of five plant virus infecting tobacco including PVY, TVBMV, TMV, TEV and CMV [16], but not including the detection of ChiVMV. In this work, we designed specific amplification primers based on the results of NGS and Sanger sequencing analysis and established a multiplex RT-PCR analysis, which is more suitable for the simultaneous detection of viruses in Sichuan tobacco planting area. Collectively, the developed multiplex RT-PCR assay was proved to be a sensitive diagnostic tool for the detection of the viruses from the leaves of naturally infected tobacco plants.

## Conclusions

In this study, we identified three kinds of potyvirus including PVY, ChiVMV and TVBMV together with TMV as well as CMV from tobacco in the field of Deyang and Luzhou city in Sichuan province using next generation sequencing of small RNAs. A multiplex RT-PCR assay was developed as an easy, fast and sensitive method for the simultaneous detection of these frequent viruses infecting cigar and flue-cured tobacco crops in Sichuan. The results of this work also provide prompt valuable disease status information for tobacco breeders.

## Abbreviations

sRNAs: Small RNAs; PVY: Potato virus Y; TVBMV: Tobacco vein banding mosaic virus; ChiVMV: Chilli veinal mottle virus; TMV: Tobacco mosaic virus; CMV: Cucumber mosaic virus; RT-PCR: Reverse transcription polymerase chain reaction; dsRNA: Double stranded RNA ; DCL: DICER-like; vsRNAs: Virus-derived small interfering RNAs; RDRs: RNA-dependent RNA polymerases; NGS: Next-generation sequencing; TEV: Tobacco etch virus; BLAST: Basic Local Alignment Search Tool; ToMV: Tomato mosaic virus; TMGMV: Tobacco mild green mosaic virus.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

### Competing interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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## Authors' contributions

TZ, SZ and XZ performed the experiments. XZ and MA participated in experimental design and coordination. YC, JW, RZ and HX collected the field tobacco samples. TZ and MA drafted the manuscript. YW, XZ and MA proofread and finalized the manuscript. All of the authors have approved the final manuscript.

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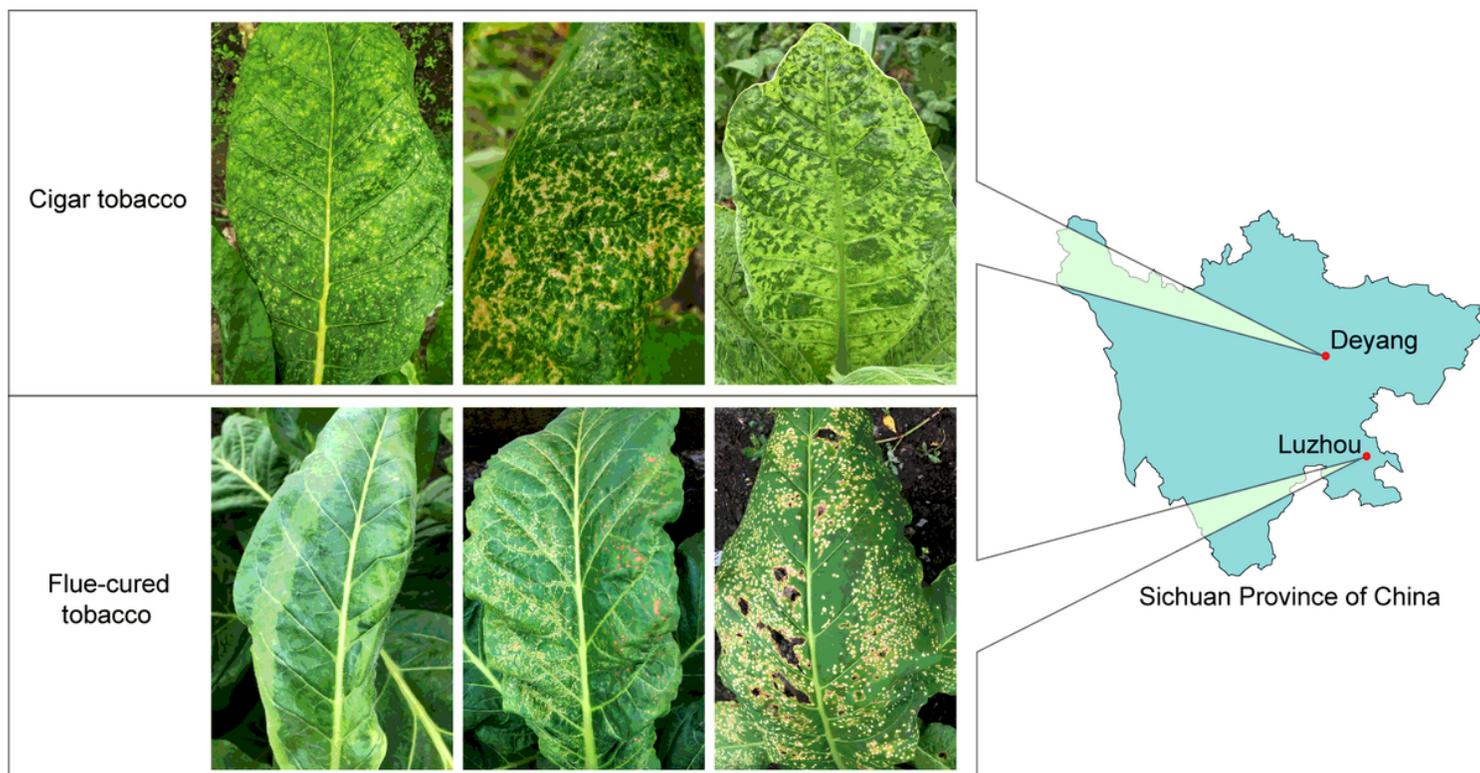
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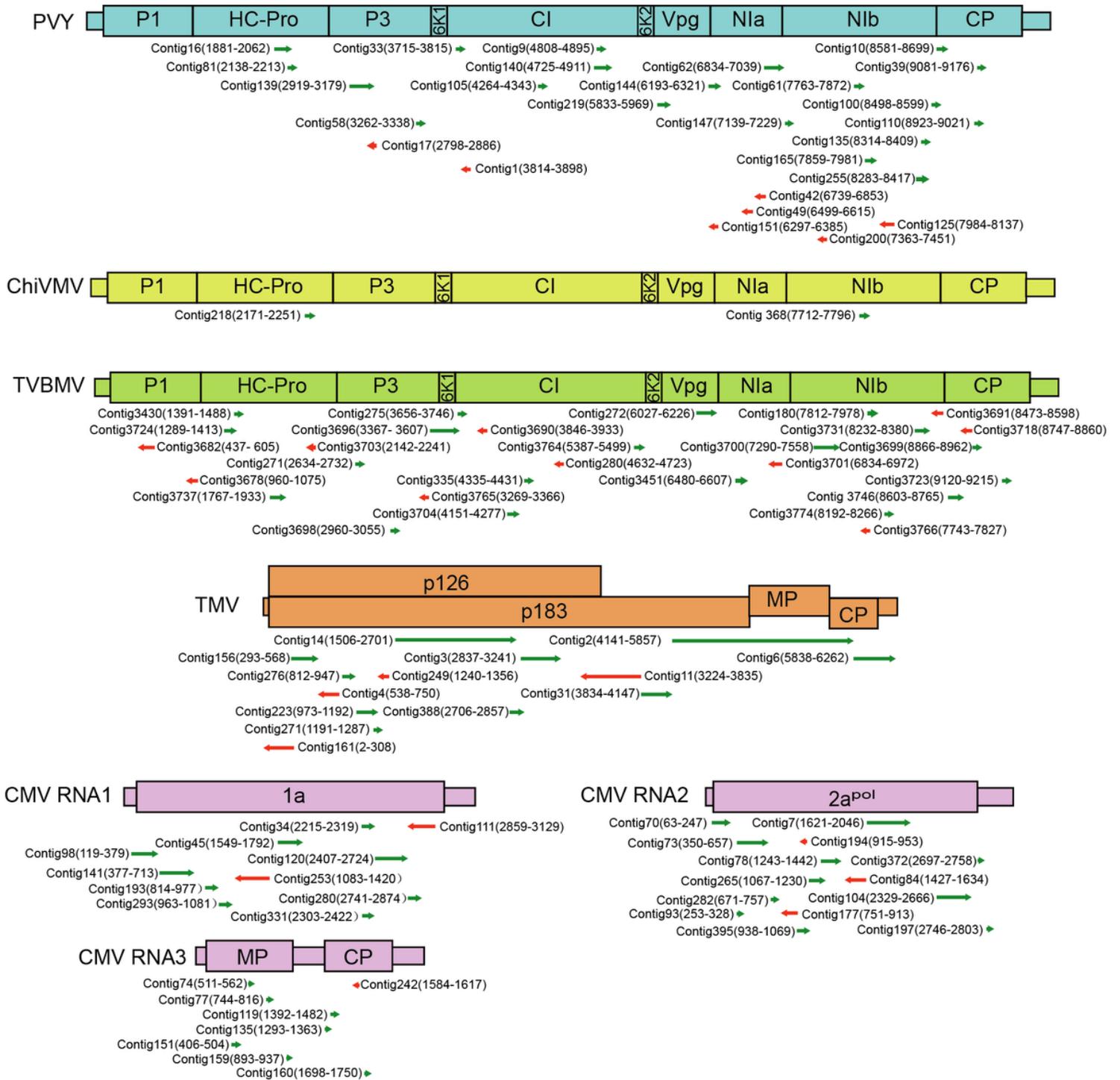
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## Figures



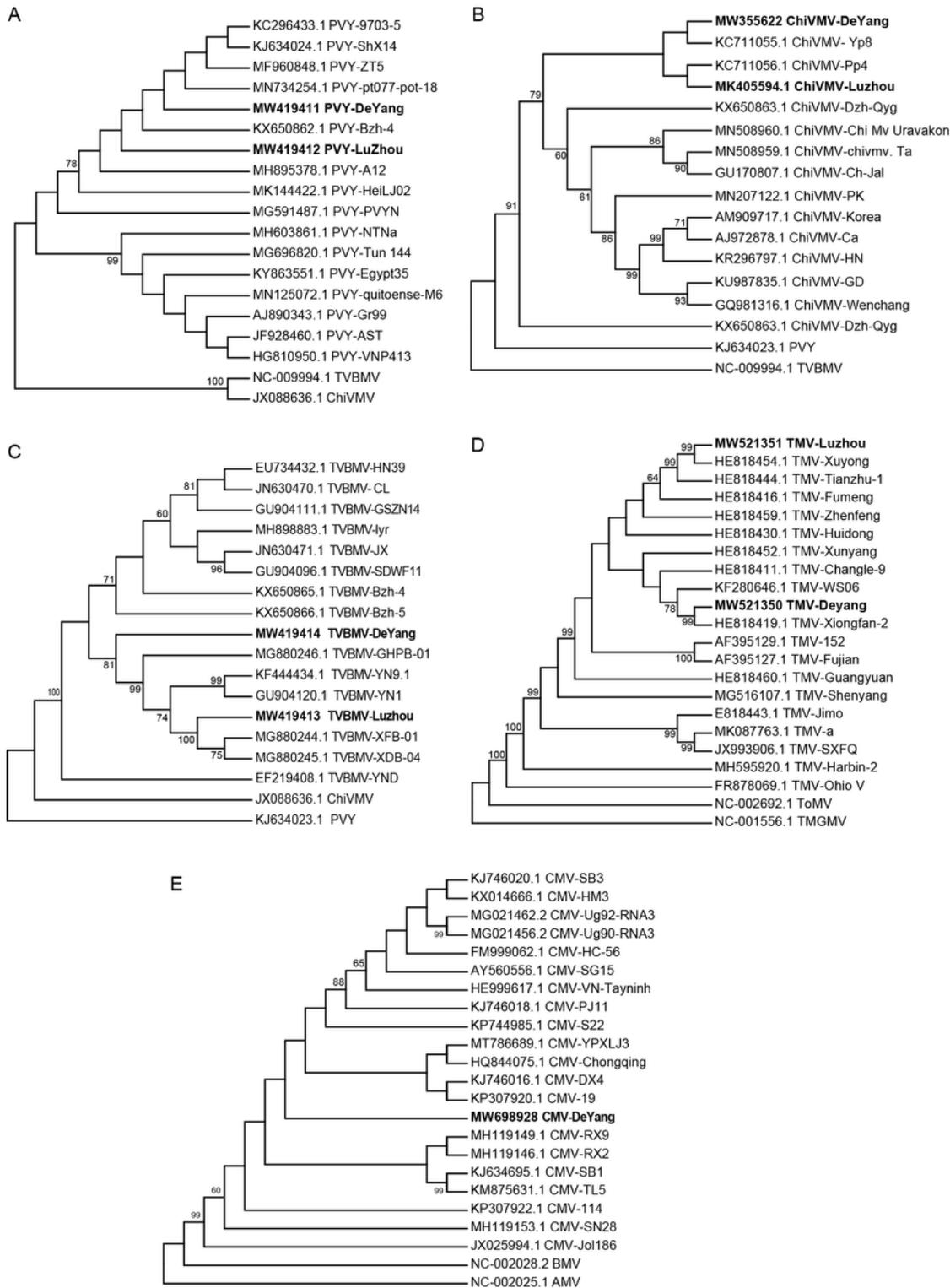
**Figure 1**

Cigar and flue-cured tobacco leaf samples with typical virus disease symptoms were collected from Deyang and Luzhou city in Sichuan Province of China. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.



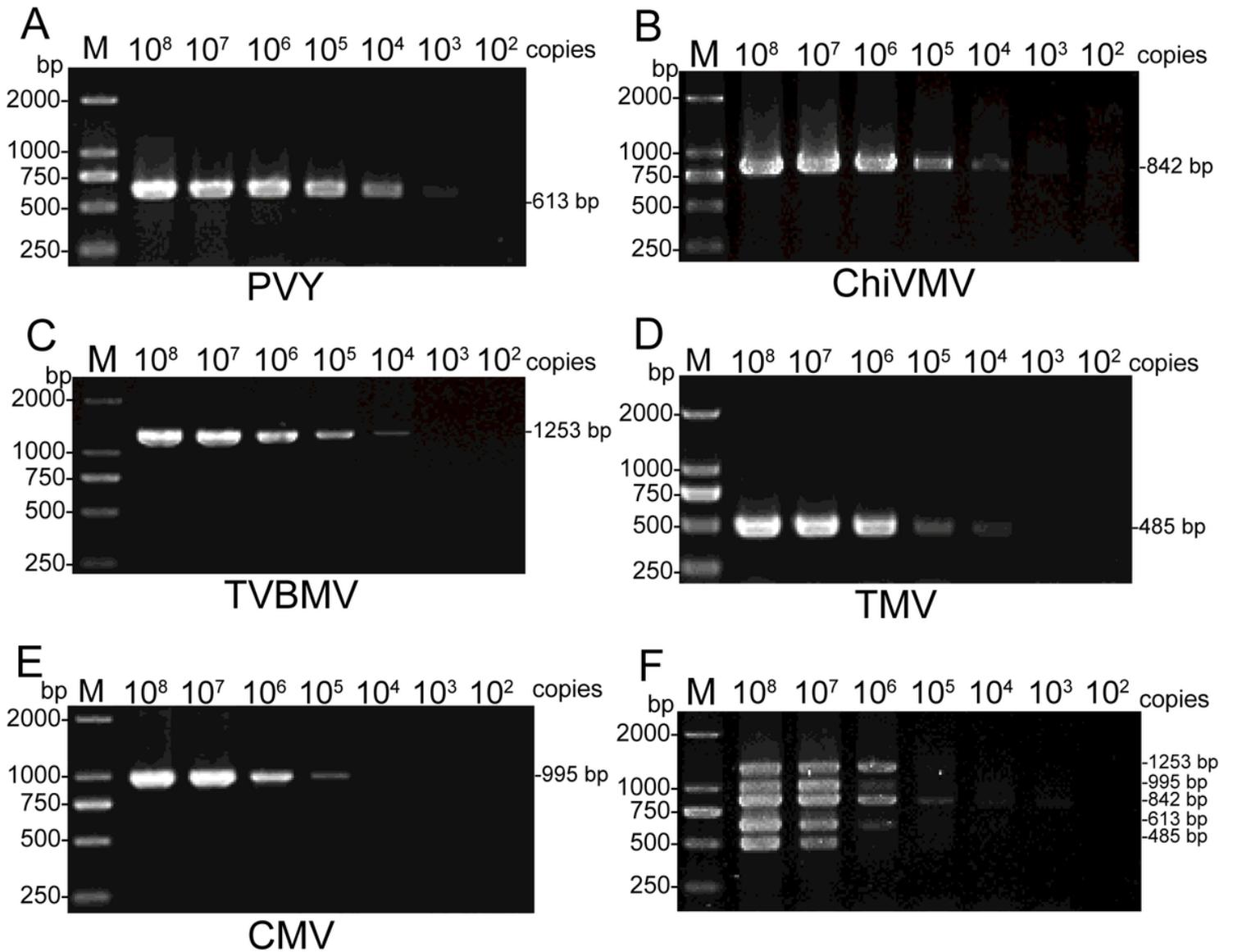
**Figure 2**

Contigs mapped to the genomes of PVY, ChiVMV, TVBMV, TMV and CMV, respectively, based on the results of high-throughput small RNA sequencing. The contigs mapped to the virus positive-strand RNA were shown in green color and those mapped to negative-strand RNA were shown in red color.



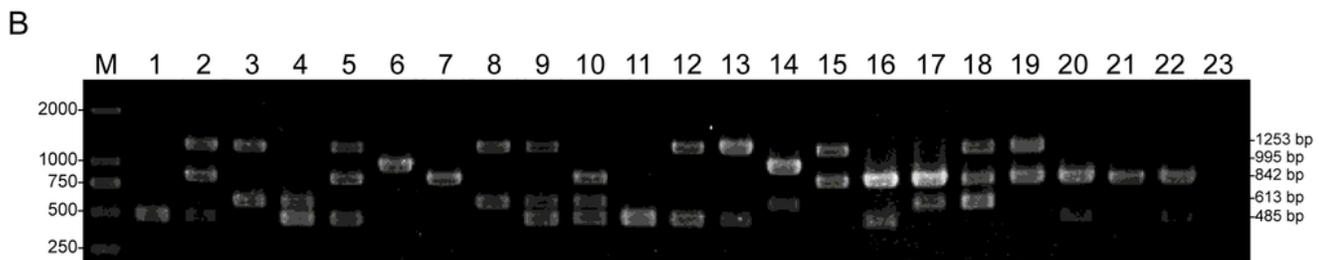
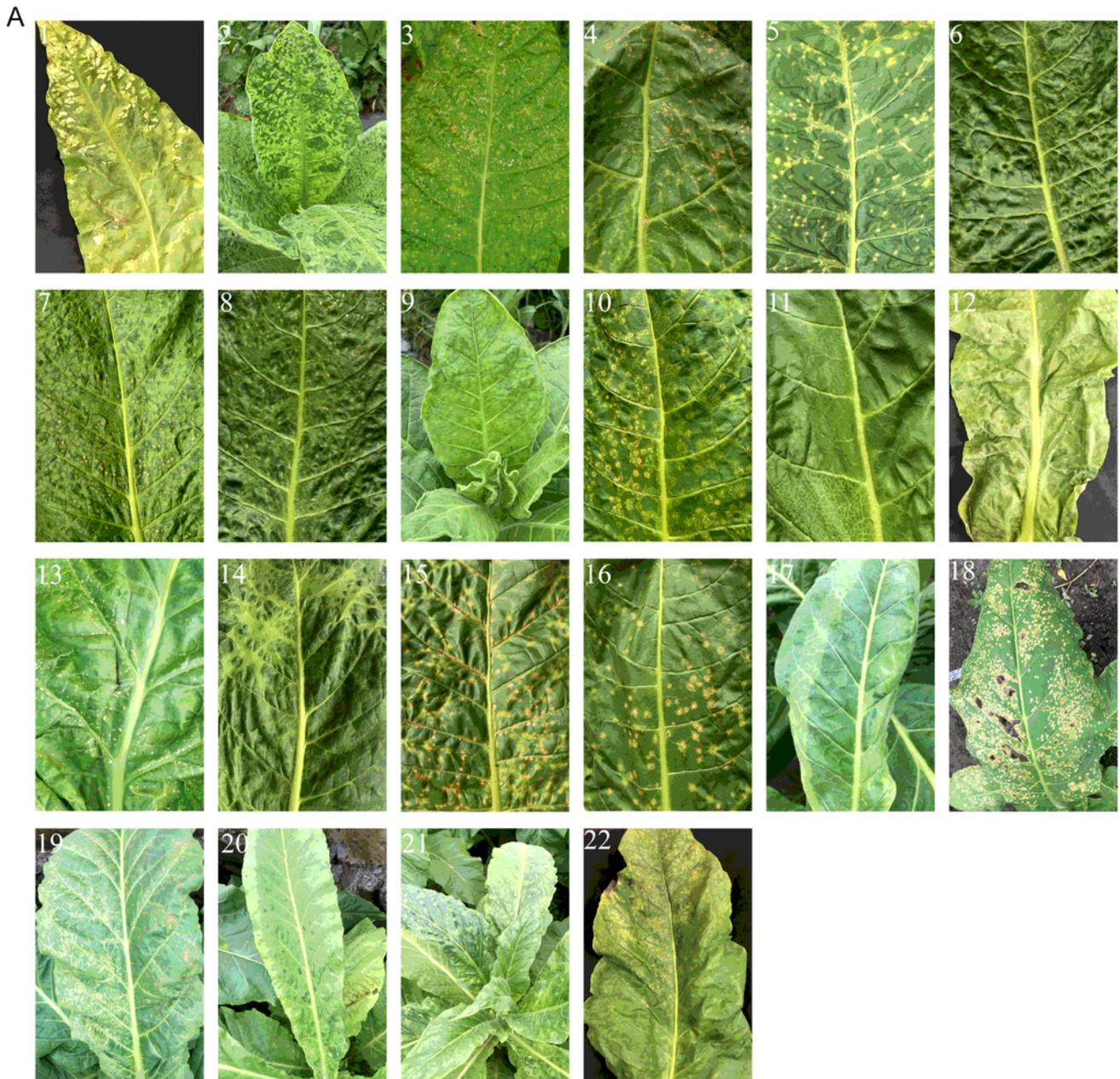
**Figure 3**

Phylogenetic analysis performed by MEGA7 based on the partial nucleotide sequences of PVY (A), ChiVMV (B), TVBMV (C), TMV (D) and CMV (E) collected from Deyang and Luzhou using Maximum Likelihood method. Numbers at the nodes of the branches represent percentage bootstrap values (1000 replicates).



**Figure 4**

Multiplex RT-PCR detection of tobacco-infecting PVY, ChiVMV, TVBMV, TMV and CMV, and its application in field sample detection. (A-E) Sensitivity and specificity of multiplex RT-PCR were tested by detecting the plasmids containing virus sequences (pEASY-PVY, pEASY-ChiVMV, pEASY-TVBMV, pCB-TMV-SY and pEASY-CMV, respectively) diluted from  $1 \times 10^8$  to  $1 \times 10^2$  copies, each. (F). Simultaneous detection of these mixed plasmids diluted from  $1 \times 10^8$  to  $1 \times 10^2$  copies using multiplex RT-PCR.



**Figure 5**

(A) Typical yellow mosaic, deformation, stunting and necrotic spot symptoms of the tobacco samples collected from Deyang (cigar tobacco leaf sample 1-17) and Luzhou (flue-cured tobacco leaf sample 18-22) city of Sichuan Province. (B) Detection of PVY, ChiVMV, TVBMV, TMV, and CMV from leaves of tobacco using multiplex RT-PCR. Lanes 1–16, leaf samples of cigar tobacco from Deyang city. 17–22, leaf samples of flue-cured tobacco from Luzhou city. Lane 23 is a negative control.

## Supplementary Files

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- [tableS1.xlsx](#)