Plant Pathol. J. 38(5) : 533-540 (2022)
https://doi.org/10.5423/PPJ.OA.04.2022.0058
eISSN 2093-9280

Research Article Open Access

The Plant Pathology Journal

Plant Virome Analysis by the Deep Sequencing of Small RNAs of *Fritillaria thunbergii* var. *chekiangensis* and the Rapid Identification of Viruses

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(Received on April 27, 2022; Revised on August 28, 2022; Accepted on August 31, 2022)

Thunberg fritillary (*Fritillaria thunbergii*), a perennial used in traditional Chinese herbal medicine, is a members of the family *Liliaceae*. The degeneration of germplasm is a severe problem in the production of *Fritillaria thunbergii* var. *chekiangensis*. However, no information about viral infections of *F. thunbergii* var. *chekiangensis* has been reported. In this study, we sequenced the small RNAs of *F. thunbergii* var. *chekiangensis* from leaves and bulbs, and viruses were identified using a phylogenetic analysis and BLAST search for sequence. In addition, multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) was used to rapidly detect viruses in this variety. Our study first reported that five viruses infected *F. thunbergii* var. *chekiangensis*. Among them, fritillary virus Y (FVY), lily mottle virus (LMoV), Thunberg fritillary mosaic virus (TFMV), and hop yellow virus (HYV) had been reported in *F. thunbergii*, while apple stem grooving virus was first reported in the genus *Fritillaria*. A multiplex RT-PCR method was developed to rapidly test the four viruses FVY, LMoV, TFMV, and HYV in *F. thunbergii* var. *chekiangensis*. Our results provide a better understanding of the infection of *F. thunbergii* var. *chekiangensis* by viruses and a basic reference for the better design of suitable control measures.

Keywords **:** *Fritillaria thunbergii* var. *chekiangensis*, multiplex RT-PCR, small RNA sequencing, virus

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Handling Editor : Seung-Kook Choi

Thunberg fritillary (*Fritillaria thunbergii*), a perennial herb in the *Liliaceae* family, is a traditional Chinese medicine and listed as one of the famous "eight flavors of Zhejiang" (Wu et al., 2021). The dry bulbs of *F. thunbergii*, called "Zhebei," are often utilized to treat coughs caused by bronchitis, inflammation, hypertension, gastric ulcers, diarrhea, bacterial infections and drug-resistant leukemia (Cui et al., 2018; Wang et al., 2013). *F. thunbergii* var. *chekiangensis* is primarily produced in Dongyang City and Pan'an County, Zhejiang Province (Li et al., 1999). The bulbs of the plant are called "Dongbei" and are generally considered as a superior product of "Zhebei" (Li et al., 1999). *F. thunbergii* var. *chekiangensis* was introduced to locations away from its indigenous area to better promote and expand its utilization (Xu and Li, 1999). However, the germplasm resources of *F. thunbergii* var. *chekiangensis* have been degenerated and endangered now. Exploring the possible mechanisms that could cause the extinction of *F. thunbergii* var. *chekiangensis* are important for the rescue and utilization of this variety.

Low level germplasm is a severe problem in the produc-

tion of *F. thunbergii* (Chen and Qian, 2002), which could be caused by viral infections in the leaves and bulbs of this species (Chen et al., 2006; Qin et al., 2006; Wei et al., 2005). Wei et al. (2005) isolated a potyvirus that causes mosaic symptoms in fresh leaves of Thunberg fritillary that were collected from fields in Ningbo City and Panan County, Zhejiang province and found it was most closely related to lily mottle virus (LMoV) (53.0% amino acid identity), leek yellow stripe virus, and lycoris mild mottle virus (72.8% nt identity in their coat protein cistrons). It was finally named Thunberg fritillary mosaic virus (TFMV) (Wei et al., 2005). Chen et al. (2006) isolated another potyvirus from fresh leaves of Thunberg fritillary and found that it was a member of the bean common mosaic virus subgroup. The most closely related species are soybean mosaic virus and Wisteria vein mosaic virus, with 68-69% amino acid identity between their polyproteins. It was finally named fritillary virus Y (FVY) (Chen et al., 2006). Thunberg fritillary was verified to be a new host of LMoV (Qin et al., 2006) and potato leaf roll virus (Qin, 2006) using reverse transcription polymerase chain reaction (RT-PCR). Recently, the amount of *F. thunbergii* var. *chekiangensis* produced has been shrinking yearly. Some detrimental trends, such as mosaic and leaf mottle symptoms and a reduction in the number of bulbs, appeared during the cultivation of *F. thunbergii* var. *chekiangensis*. Although *F. thunbergii* has been studied, it was unclear whether *F. thunbergii* var. *chekiangensis* was infected by these viruses until this study.

Plant and invertebrates can protect themselves from viral infection through RNA silencing, which is induced by small RNAs (sRNAs) that are 21-30 nucleotides long (Ding and Voinnet, 2007). Recently, virome analysis based on deep sRNA sequencing has been developed to identify and detect viruses in plants without any previous knowledge of their viral genomes (Bi et al., 2012; Golyaev et al., 2019; Massart et al., 2017; Roossinck et al., 2015; Singh et al., 2020). In addition, virome analyses based on deep sRNA sequencing are 10-fold more sensitive than those that utilize RT-PCR (Santala and Valkonen, 2018). However, there has not been a comprehensive study using virome analysis based on deep sRNA sequencing for *F. thunbergii* and its varieties.

In this study, we sequenced the sRNAs of *F. thunbergii* var. *chekiangensis* from leaves and bulbs and identified the viruses using phylogenetic analyses and BLAST search for sequence. In addition, a multiplex RT-PCR was used to rapidly detect viruses in the different tissues of *F. thunbergii* var. *chekiangensis*. To our knowledge, this study is the first to reveal viral populations in *F. thunbergii* var. *chekiangensis* and provides a multiplex RT-PCR method to rapidly detect viruses in this variety.

Materials and Methods

Plant materials. *F. thunbergii* var. *chekiangensis* was cultured in the main original cultivation basement in Houge Village, Pan'an County, Zhejiang Province, China (120″36′09E, 28″57′56N). The area is a humid subtropical monsoon climate with an annual mean temperature from 13.9°C to 17.4°C and annual mean precipitation from 1,409.8 mm to 1,527.8 mm. The leaves and bulbs of *F. thunbergii* var. *chekiangensis* were collected in April 2021. Leaves and bulbs from three individuals were collected as three biological replicates. After collection, all the samples were frozen in liquid nitrogen and stored at –80°C until further analysis.

Extraction of total RNA. Total RNA from leaves and bulbs was extracted using an RNAprep Pure Plant Kit (Tiangen Biotech Co., Ltd., Beijing, China). The purity of total RNA was determined using a NanoPhotometer (Implen, Inc., Westlake Village, CA, USA), and the content of total RNA was determined using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). The integrity of total RNA was evaluated using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

Library construction and sequencing. The sRNA libraries were constructed using an NEB Next Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs Inc., Ipswich, MA, USA) following the manufacturer's instructions. Schematic diagram was shown in Supplementary Fig. 1. Index codes were added to attribute the sequences to each sample. Six sRNA libraries of leaves and bulbs from three plants were constructed. An NEB 3' SR Adaptor was directly and specifically ligated to the 3' end of microRNA, small interference (siRNA) and PIWI-interacting RNA. After the 3' ligation reaction, the SR RT Primer hybridized to the excess of 3' SR Adaptor that remained free after the 3' ligation reaction transformed the single-stranded DNA adaptor into a double-stranded DNA molecule. mRNAs with a poly-A tail were extracted using poly-T oligo-attached magnetic beads. The purified mRNAs were subjected to the first strand of cDNA followed by a second strand of cDNA, and the 3′ ends were adenylated. The first strand cDNA was then synthesized using M-MuLV Reverse Transcriptase (RNase H^-) (New England Biolabs Inc.). LongAmp Taq 2× Master Mix (New England Biolabs Inc.), SR Primer for Illumina and index (X) primer were utilized to amplify the fragments. The RT-

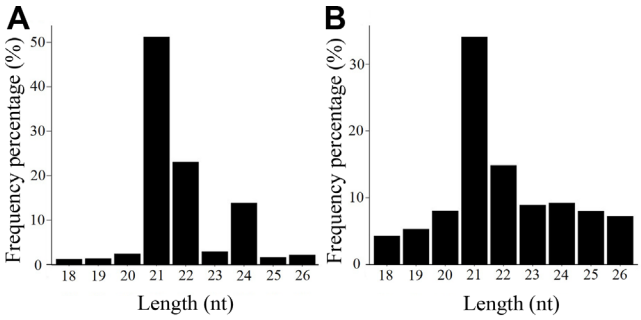

Fig. 1. Distribution of reads with different length screened from the bulb (A) and leaf (B) libraries of *Fritillaria thunbergii* var. *chekiangensis*.

PCR products were purified, and the DNA fragments that corresponded to 140-160 bp (the length of small noncoding RNA plus the 3' and 5' adaptors) were recovered and dissolved in elution buffer. Finally, the quality of library was assessed on an Agilent Bioanalyzer 2100 system (Agilent Technologies). The clustering of these samples with an adapter was performed on a cBot Cluster Generation System using a Tru Seq SR Cluster Kit v3-c Bot-HS (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. After cluster generation, Novogene Corporation, Inc. (Beijing, China) sequenced the RNA on an Illumina NovaSeq 6000 platform, which generated 50 bp single end reads. All the raw data were submitted to the NCBI database with the accession number of SRR18739256 and SRR18739255.

Bioinformatics analysis. Raw reads of the FASTA format were first processed using custom Perl and Python scripts. Clean reads were obtained by removing the reads that contained poly N, with 5′ adapter contaminants, without a 3′ adapter or insert tag, poly A or T or G or C and low-quality reads from raw data. The plants that are infected with viruses accumulate high levels of viral siRNAs of the three major size classes: 21-nt, 22-nt, and 24-nt (Aregger et al., 2012). Size ranges from 18- to 26-nt was selected from the clean reads for further research. sRNA reads 18- to 26-nt long were assembled into contigs using Velvet 1.2.10 (https://www.ebi.ac.uk/~zerbino/velvet/) and SPAdes 3.15.3 (http://cab.spbu.ru/software/spades/) to identify the viruses. The contigs from these two types of software were combined and processed with Cap3 (http://doua.prabi.fr/software/cap3). The contigs were blasted on the NCBI nucleotide BLAST database (BLASTN and BLASTX), and the annotated information of viruses related to contigs was screened to obtain the candidate virus list. The most closely related reference sequences of the viral species were analyzed in more detail. The candidate virus list was further corrected by mapping the 18- to 26-nt sRNA reads to the database composed of screened virus nucleic acid sequences using Bowtie 2 (Langmead and Salzberg, 2012) with up to 1 mismatch.

Multiple sequence alignment and phylogenetic analysis. Phylogenetic trees were constructed using Clustal X (version 2.1) (http://www.clustal.org/clustal 2/) with default parameters and the maximum likelihood method in MEGA 7.0 (http://www.megasoftware.net/). The reliability of branches was inferred from a bootstrap analysis of 1,000 replicates. The final phylogenetic tree was then edited using ITOL (http://itol.embl.de/).

Validation of viruses using RT-PCR and sequencing. Five primer pairs were designed to confirm the results of sRNA-seq based on references and contig sequences by conducting RT-PCR in different leaf and bulb samples (Supplementary Table 1). The cDNA was amplified from

Table 1. Primers used for multiplex RT-PCR and the size of products

Virus	Primer name	Sequences (5'-3')	Size of product (bp)
TFMV	TFMV5831-6409F	GCAAAGAATAAACGTCAGAGACAGA	579
	TFMV5831-6409R	TTCAAACACTAAACTCTCGGTTTCTTTCT	
LMoV	LMoV1083-2456F	TCGGGCCCAGGCGATAAATT	1,374
	LMoV1083-2456R	GCCGACCTGGTAGTGCTTCA	
FVY	FVY6305-7033F	AGCAGGTCAGTTTACAAAGGACT	729
	FVY6305-7033R	CTGGACTGCCACTGTGTCAC	
HYV	HYV-F	TTGACGACAGACTACGATCTTCCATTG	250
	HYV-R	ACTCGGTAACGCAACTCAGAACATC	
ASGV	ASGV4787-5749F	ATGGCTATCGTCAACGTCAACC	963
	ASGV4787-5749R	TCAGGGGGAGGAACCGTCAG	

RT-PCR, reverse transcriptase-polymerase chain reaction; TFMV, Thunberg fritillary mosaic virus; LMoV, lily mottle virus; FVY, fritillary virus Y; HYV, hop yellow virus; ASGV, apple stem grooving virus.

the total RNA using a TIANScript II RT Kit (Tiangen Biotech Co., Ltd.) following the manufacturer's instructions with conditions of 25°C for 10 min, 42°C for 60 min, 70°C for 15 min, and storage at 4°C. The cDNA was used as template for amplification. The RT-PCR conditions were 98°C for 30 s, followed by 30 cycles at 98°C for 10 s, 65°C to 68°C for 30 s (the annealing temperature is listed in Supplementary Table 1), and 72°C for 1 min, with a final extension at 72°C for 10 min. We checked the amplified RT-PCR products by 1.0% gel electrophoresis. The RT-PCR product was sequenced by Sangon Shanghai Co., Ltd. (Shanghai, China). The sequence was blasted on the NCBI nucleotide BLAST database to test the similarity between viruses.

Rapid detection of viruses using a multiplex RT-PCR assay. To enable the rapid identification of viruses in *F. thunbergii* var. *chekiangensis*, we designed five RT-PCR primer pairs to conduct multiplex RT-PCR assay. Detail of the primer pairs are shown in Table 1. We used the same total RNA from the pooled samples as a template, and the cDNA was amplified as described above. The RT-PCR conditions were 98°C for 30 s, followed by 30 cycles at 98°C for 10 s, 66°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. To ensure the absence of contaminants, each RT-PCR run included a negative (water) control. We checked the amplified RT-PCR products by gel electrophoresis.

Results

Overview of sRNA-seq. A total of 13,779,494 and 11,850,879 clean reads were generated from the bulb and leaf libraries, respectively, having base quality scores ≥ Q30 (Table 2). Size ranges from 18- to 26-nt were selected, and 12,269,337 and 7,071,885 reads were screened from the bulb and leaf libraries, respectively. Among them, 21-nt reads were the most abundant (51.26% and 34.1% in the bulbs and leaves, respectively), followed by 22-nt (23.11% and 14.86% in the bulbs and leaves, respectively) (Fig. 1).

Clean reads from two libraries were individually assembled using SPAdes and Velvet. A total of 267 and 120 contigs were obtained from the bulb and leaf libraries when assembled by SPAdes, and 383 and 419 contigs were obtained when assembled by Velvet, while 354 and 201 contigs were obtained when they were both merged (Table 2).

Phylogenetic relationship of the contigs. The assembled contigs were annotated and compared with the virus reference database in GenBank using BLAST. Apple stem

Table 2. Length distribution of assembled contigs in the libraries of bulbs and leaves of *Fritillaria thunbergii* var *chekiangensi*

Library	Software	No. of contigs	0-200 bp	200-500 bp	500-1k bp	1k-2k bp	>2k bp	N50	N90
Bulbs	SPAdes	267	0	258	6	1	2	248	74
Leaves	SPAdes	120	0	95	17	5	3	613	207
Bulbs	Velvet	383	172	153	38	15	5	440	147
Leaves	Velvet	419	159	185	48	23	4	486	166
Bulbs	Merge[a]	354	243	77	24	7	3	526	124
Leaves	Merge[a]	201	80	72	26	18	5	793	201

[a]The contigs from two types of software were combined.

Table 3. Viruses identified from the viromes in the bulbs and leaves of *Fritillaria thunbergii* var chekiangensi using small RNA (sRNA) deep sequencing and the number of contigs

Organs	Family/Genus	NCBI reference	Annotation	No. of contigs	E-value
Leaf	*Potyviridae/Potyvirus*	AM039800.1	Fritillary virus Y	18	2.50426E-37
		MT795719.1	Lily mottle virus	21	4.50283E-49
		NC007180.1	Thunberg fritillary mosaic virus	26	1.39101E-10
	Bromoviridae/Anulavirus	MN520242.1	Hop yellow virus	7	4.2216E-10
	Betaflexiviridae/ Capillovirus	KR185346.1	Apple stem grooving virus[a]	32	1.4197E-07
		MH108979.1	Citrus tatter leaf virus[a]		
Bulb	*Potyviridae/Potyvirus*	AM039800.1	Fritillary virus Y	1	1.28E-07
		MT795719.1	Lily mottle virus	26	5.62875E-07
	Bromoviridae/Anulavirus	NC007180.1	Hop yellow virus	12	1.28E-07

[a]Both of species are the potential virus in the leaves of *F. thunbergii* var *chekiangensi* based on the data of sRNA sequencing.

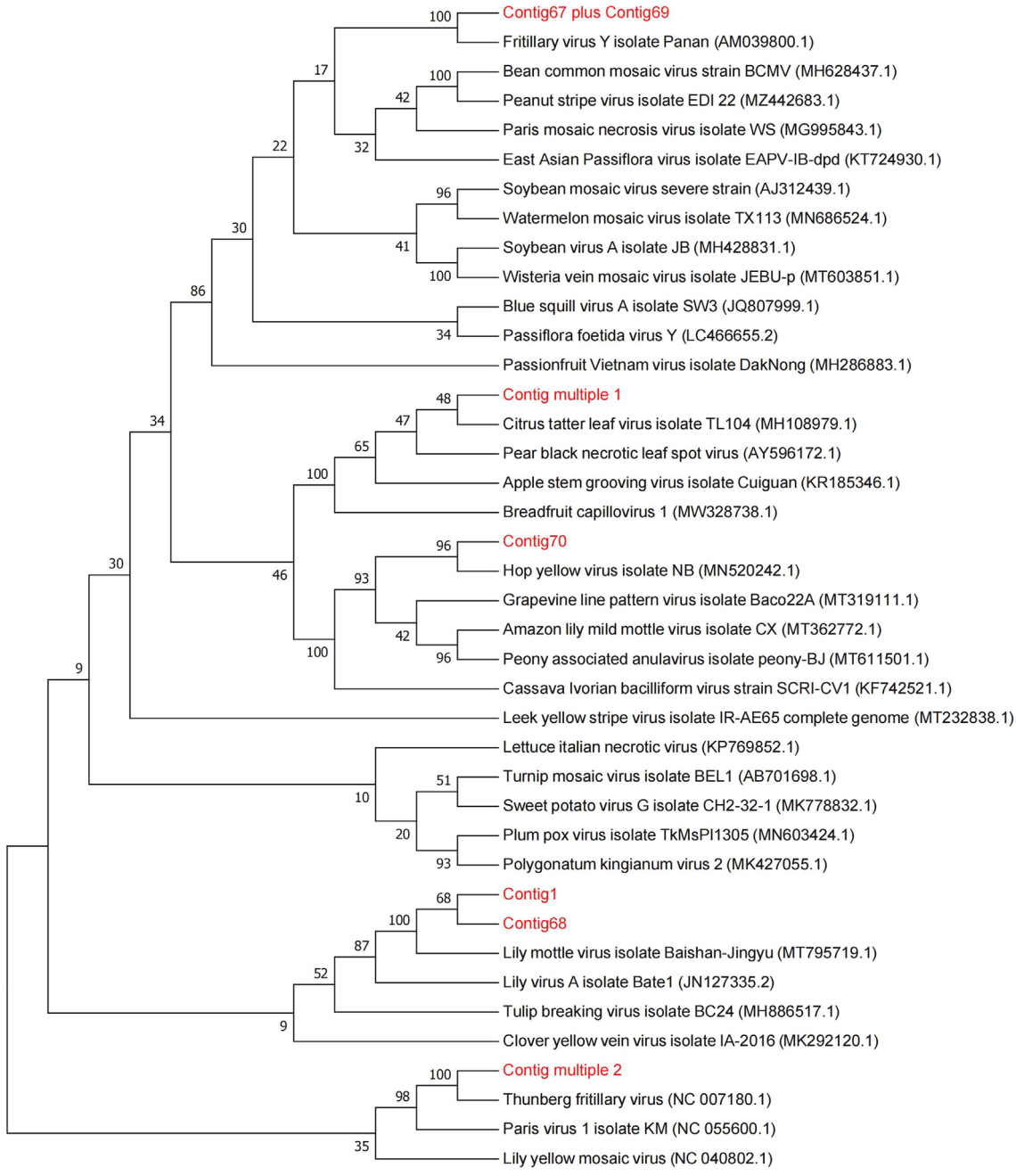

Fig. 2. Phylogenetic tree reconstruction using the neighbor-joining method based on alignment.

grooving virus (ASGV) or citrus tatter leaf virus (CTLV) (32 contigs) was the dominant virus that infected *F. thunbergii* var. *chekiangensis* followed by LMoV (26 contigs) and TFMV (26 contigs) (Table 3).

In the phylogenetic tree using the contigs from bulb and leaf libraries (Fig. 2), contig67 plus contig69 which was concatenated by two assembled contigs, was 100% similar to FVY (AM039800.1). Contig1 and contig68 were both 100% similar to LMoV (MT795719.1). Contig70 was 98.28% similar to hop yellow virus (HYV, MN520242.1). Contig multiple 1 (Fig. 3A) was 97.25% similar to CTLV (MH108979.1), 92.86% similar to pear black necrotic leaf spot virus (PBNLSV; AY596172.1), 95.60% similar with ASGV (KR185346.1), and 84.70% similar to breadfruit capillovirus 1 (BCV1; MW328738.1). Fig. 3B indicates 100% similarity to TFMV (NC007180.1).

Identification of viruses. The results of RT-PCR of

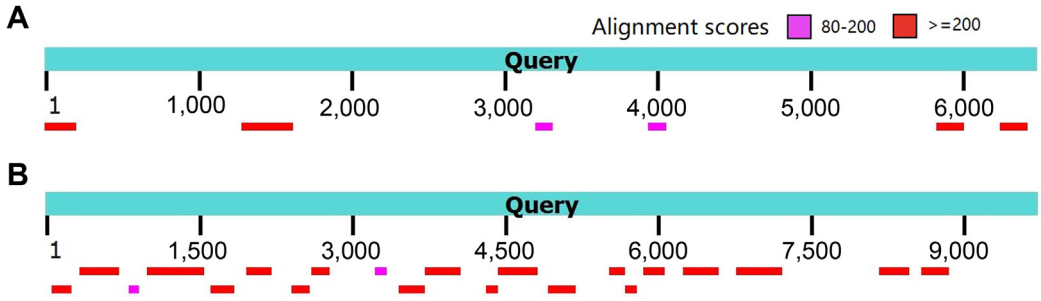

Fig. 3. (A) Contig multiple 1, which include 6 contigs. (B) Contig multiple 2, which include 21 contigs.

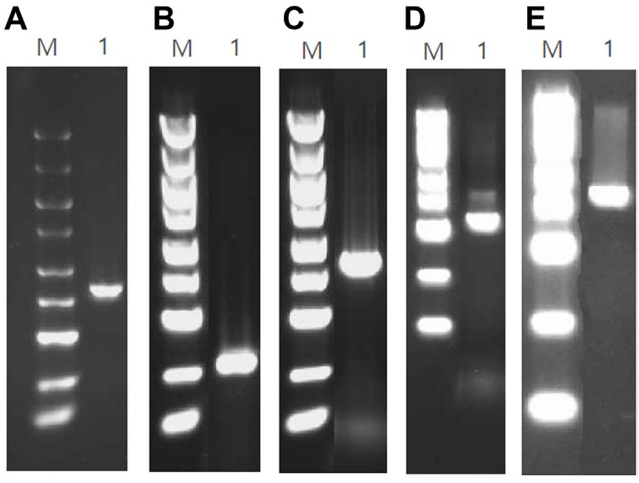

Fig. 4. Representative results for the detection of lily mottle virus (A), hop yellow virus (B), fritillary virus Y (C), apple stem grooving virus (D), and Thunberg fritillary mosaic virus (E) by reverse transcriptase-polymerase chain reaction (RT-PCR). M, DNA marker (100-5,000 bp); lane 1, RT-PCR product. The size of RT-PCR products from (A) to (E).

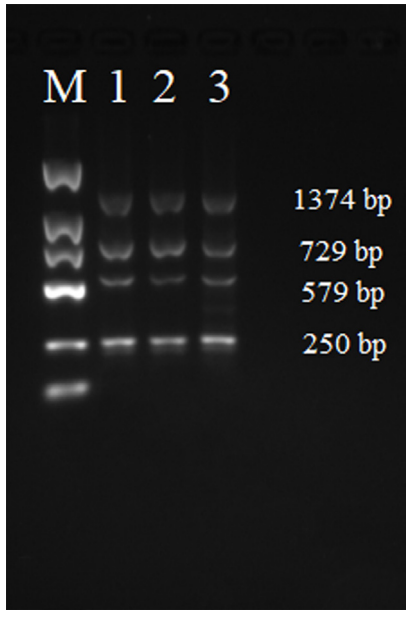

Fig. 5. Representative results to detect fritillary virus Y, lily mosaic virus, Thunberg fritillary mosaic virus, and hop yellow virus by multiplex reverse transcriptase-polymerase chain reaction (RT-PCR). M, DNA marker (100-2,000 bp); lane 1-3, RT-PCR products.

the five viruses are shown in Fig. 4. All the primer pairs yielded amplicons of the expected size. The sequences of the RT-PCR products of "FVY", "TFMV", and "LMoV" were 99.12%, 98.08%, and 98.50% identical to those of FVY (AM039800.1), TFMV (HM584812.1), and LMoV (MK368802.1), respectively. The sequences of the RT-PCR product of "HYV" were 98.49% identical with that of HYV (MN520242.1) and 94.92% identical with that of grapevine line pattern virus (MW888442.1). The sequence of the RT-PCR product "ASGV" was 94.40% identical with ASGV (LC143387.1) and 94.18% with CTLV (MH108979.1).

Based on the evidence, five and three viruses were identified in the leaves and bulbs, respectively (Table 3). Among them, FVY, LMoV, and TFMV are species in the genus *Potyvirus* of the family *Potyviridae*. HYV is a species in the genus *Anulavirus* of the family *Bromoviridae*. ASGV is a species in the genus *Capillovirus* of the family *Betaflexiviridae*, and alternatively, CTLV is considered as a strain of ASGV (Tatineni et al. 2009).

Rapid detection by multiplex RT-PCR. Multiplex RT-PCR was used to rapidly detect multiple viruses in one reaction. After primer design and condition optimization, multiplex RT-PCR with the amplification of four primer pairs succeeded (Table 1). Four RT-PCR fragments with the expected size are shown in Fig. 5, indicating that the multiplex RT-PCR identified the four viruses FVY, LMoV, TFMV, and HYV in *F. thunbergii* var. *chekiangensis*. Unfortunately, primer pairs for ASGV could not be amplified in the multiplex RT-PCR experiment.

Discussion

High-throughput sequencing technology and bioinformatics analysis of the sRNA population isolated from plants allows universal virus detection. For example, Singh et al. (2020) confirmed the presence of three known viruses, and also confirmed the first report of cereal yellow dwarf virus and wheat yellow dwarf virus in wheat using the de novo assembly of high-quality contigs. In our study, 143 contigs were identified in *F. thunbergii* var *chekiangensi* through sRNA-seq data mining. To our knowledge, this is the first report of FVY, LMoV, TFMV, HYV, and ASGV (or CTLV) in *F. thunbergii* var. *chekiangensis*. FVY, LMoV, TFMV, and HYV were reported from *F. thunbergii* (https://www.ncbi.nlm.nih.gov/nuccore/MN520242.1/) (Chen et al. 2006; Qin et al. 2006; Wei et al. 2005), while ASGV (or CTLV) was first reported in the genus *Fritillaria*.

At first, LMoV was considered to only spread among lilies (Asjes, 2000). However, *F. thunbergii* was later verified as a host of LMoV (Qin et al., 2006). TFMV is a new potyvirus found in the leaves of *F. thunbergii* in both Ningbo and Pan'an cities, Zhejiang Province, China (Wei et al., 2005). The leaves of most plants display mottle and mosaic symptoms after infection (Wei et al., 2005). FVY is a potyvirus that coexists with TFMV in the leaves of *F. thunbergii* in Ningbo and Panan cities, Zhejiang Province, China (Chen et al., 2006). However, FVY is not widespread in the fields of *F. thunbergii*, and only 22.85% of the plants were infected with FVY (Chen et al., 2006). In our study, the above three viruses were found in the leaves of *F. thunbergii* var. *chekiangensis*, while only LMoV and FVY were found in the bulbs of this variety.

HYV is a tentative member of the genus *Anulavirus* of the family *Bromoviridae*. The complete sequence of HYV isolated from *F. thunbergii* was accepted in NCBI (Lu Y, unpublished data). However, no other information on this virus is currently available. In our study, the contig70 was 98.276%, 93.863%, and 93.636% identical with those of HYV (MN520242.1), Amazon lily mild mottle virus (ALiMMV) (MT362772.1), and grapevine line pattern virus (GLPV) (MT309111.1), respectively. The RT-PCR product and HYV (MN520242.1) had the highest degree of homology (98.49%). The presence of HYV in *F. thunbergii* (Lu Y, unpublished data) led us to hypothesize that the virus can be found in *F. thunbergii* var. *chekiangensis*. However, more studies are needed to verify the characterization of HYV in *F. thunbergii* var. *chekiangensis.*

In our study, the contig multiple 1 was 97.253% similar to CTLV and 95.604% similar to ASGV (KR185346.1). However, the primer pairs designed based on the sequence of ASGV obtained an amplicon of the expected size, and the RT-PCR product was 94.40% identical with ASGV (LC143387.1) and 94.18% with CTLV. Tatineni et al. (2009) considered that CTLV is a strain of ASGV. Song et al. (2015) reported that CTLV may be not a distinct strain of ASGV, and proposed that CTLV should be renamed as ASGV following a complete genome sequence analysis of two CTLV isolates from China. Further studies, including the isolation of virus and the amplification of the complete sequences, are needed to confirm the presence of ASGV or CTLV in the genus *Capillovirus* (*Betaflexiviridae* family) in *F. thunbergii* var. *chekiangensis.*

The multiplex RT-PCR assays can help to save time and costs in testing for multiple viruses. Liu et al. (2018) developed multiplex RT-PCR primers to successfully detect six respiratory viruses using TaqMan probes in a single reaction. Menzel et al. (2002) found that the multiplex RT-PCR assays described are reliable, rapid, and sensitive methods to detect these viruses and may replace techniques that commonly require indexing by woody indicators or enzyme-linked immunosorbent assay. In our experiment, we designed the RT-PCR primer pairs, optimized the conditions for PCR, and found that the multiplex RT-PCR identified the four viruses FVY, LMoV, TFMV, and HYV in *F. thunbergii* var. *chekiangensis,* although it did not detect ASGV.

Our results are the first report of the identification of five viruses in *F. thunbergii* var. *chekiangensis* using the sRNA deep sequencing method. Among them, FVY, LMoV, TFMV, and HYV had been reported in *F. thunbergii*, while ASGV was first reported in the genus *Fritillaria*. Further studies are needed to confirm the presence of ASGV or a new virus in the genus *Capillovirus* (*Betaflexiviridae* family) in *F. thunbergii* var. *chekiangensis.* A multiplex RT-PCR method was developed to rapidly test four viruses of FVY, LMoV, TFMV, and HYV in *F. thunbergii* var. *chekiangensis.* Our results should provide a better understanding of the infection of *F. thunbergii* var. *chekiangensis* by viruses, and thus, enable the design of suitable control measures, which can provide technical support for the culture and development of *F. thunbergii* var. *chekiangensis* in the future.

Conflicts of Interest

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

Acknowledgments

This work was carried out with the support of the demonstration project of traditional Chinese medicinal green

development in Pan'an County (202004), China.

Electronic Supplementary Material

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

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