Characterization of a New Orthotospovirus from Chilli Pepper in Yunnan Province, China

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Abstract

Chilli pepper (*Capsicum annuum* L.) is one of the most important crops in Yunnan Province, China. An orthotospovirus isolate 14YV855 was isolated from a diseased chilli pepper plant exhibiting yellow ringspots and necrosis on leaves in Shiping County, Honghe Hani and Yi Autonomous Prefecture, Yunnan Province in 2014. The complete genome sequence of 14YV855 was determined. The small, medium, and large RNAs are 3,428, 4,781, and 8,917 nucleotides long, respectively. The complete nucleocapsid (N) protein of 14YV855 shares a high amino acid identity of 84.8 to 89.9% to that of *Capsicum chlorosis virus* (CaCV), *Groundnut bud necrosis virus* (GBNV), *Watermelon bud necrosis virus* (WBNV), and *Watermelon silver mottle virus* (WSMoV), which is slightly less than the 90% identity threshold for the demarcation of

new *Orthotospovirus* sp. Phylogenetic analyses revealed that the N protein and RNA-dependent RNA polymerase of 14YV855 are the most related to WSMoV, while the NSs, NSm, and Gn/Gc proteins are similar to those of GBNV. As expected, 14YV855 is serologically related to CaCV, GBNV, WBNV, and WSMoV when the monoclonal antibody against the N protein of WSMoV was used; however, 14YV855 can be distinguished from other orthotospoviruses by reverse-transcription PCR using the specific primers. Our results indicate that 14YV855 is a new *Orthotospovirus* sp. belonging to the WSMoV serogroup and is provisionally named Chilli yellow ringspot virus.

Keywords: genome, orthotospovirus, phylogeny

Viruses belonging to the genus *Orthotospovirus* (family *Tospoviridae*, order *Bunyavirales*) are transmitted by thrips in a persistent, propagative manner and usually cause severe symptoms such as ringspots and necrotic spots on the leaves and apical meristem death of infected plants (Abudurexiti et al. 2019; Pappu et al. 2009). The disease caused by orthotospovirus infections leads to serious economic losses in many important cucurbitaceous and solanaceous crops worldwide (Pappu et al. 2009). Orthotospoviruses possess quasi-spherical enveloped particles of approximately 80 to 120 nm in diameter and a tripartite genome consisting of large (L), medium (M), and small (S) segments of single-stranded RNA molecules. All orthotospoviral RNA segments have a conserved terminal nucleotide sequence 5'-AGAGCAAU-3' at the 5' end and a complementary sequence 5'-AUUGCUCU-3' at the 3' end that can tightly couple with RNA-dependent RNA polymerase (RdRp) and nucleocapsid

(N) protein to form noncovalently closed pseudocircular structures (Plyusnin et al. 2012). The RdRp is encoded on the viral complementary (vc)-strand of the L segment. Both M and S segments have an ambisense coding strategy and each consists of two open reading frames (ORFs) flanked by an A-U rich intergenic region (IGR) that can form a stable hairpin structure. The M segment encodes the cell-to-cell movement protein NSm from the viral (v)-strand and the two envelope glycoproteins Gn and Gc from the vc-strand. The S segment encodes an RNA silencing suppressor NSs protein in the v-sense and the RNA-associated N protein in the vc-sense (Plyusnin et al. 2012; Tsompana and Moyer 2008).

Officially, orthotospoviruses are classified based on particle morphology, genome organization, vector specificity, plant host range, and the serological relationship of polyclonal antibodies raised against the N protein; and the amino acid sequence identity of the N protein must be lower than 90% when compared with that of previously described *Orthotospovirus* spp. (Plyusnin et al. 2012). The serological relationship of orthotospoviruses has been demonstrated to be in synchrony with their phylogenetic clustering (Chen et al. 2010; Chen et al. 2014; Kang et al. 2014; Seepiban et al. 2011). Currently, orthotospoviruses are divided into six phylogenetic clades using the N protein coding sequences, with the main geographically based origin for each clade (Turina et al. 2016). These clades have *Groundnut yellow spot virus* (GYSV), *Iris yellow spot virus* (IYSV), *Lisianthus necrotic ringspot virus* (LNRV), *Soybean vein necrosis virus* (SVNV), *Tomato spotted wilt virus* (TSWV), and *Watermelon silver mottle virus* (WSMoV) as type members. Orthotospoviruses primarily found in Asia belong to the GYSV, LNRV, IYSV, and WSMoV clades while viruses placed in the TSWV and SVNV clades mainly emerged from American and European countries (Chen and Jan 2015).

Several serological and molecular diagnostic techniques have been developed, making the identification of orthotospoviruses easier. In particular, the genus-universal degenerate primers are the most convenient tool to explore orthotospoviruses (Chen et al. 2012; Chu et al.

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2001; Cortez et al. 2001; Huang et al. 2018; Liu et al. 2017). At least 11 orthospoviruses are present in China, and 4 of them are tentative new species; namely, Hippeastrum chlorotic ringspot virus (HCRV) (Dong et al. 2013), Mulberry vein banding associated virus (MVBaV) (Meng et al. 2015), Tomato necrotic spot associated virus (TNSaV) (Yin et al. 2014), and Tomato zonal spot virus (TZSV) (Dong et al. 2008). Orthospoviruses, therefore, have become a problem for the cultivation of economic crops in China.

In November 2014, symptoms of an orthospovirus-like disease were observed on chilli pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*) in the Yunnan Province of China. The symptoms included yellow ringspots, necrotic spots, and mottling of leaves, which appeared at a low incidence of approximately 2%. A virus isolate was obtained by three local lesion transfers on leaves of *Chenopodium quinoa* from a diseased chilli sample and then denoted 14YV855. The isolate was characterized by determining its complete genome sequence, host range, and serological relationships

with other orthospoviruses. The identity of 14YV855 is demonstrated in the present study.

Materials and Methods

Virus isolation. The diseased chilli pepper sample showing yellow ringspots and necrotic spots on leaves (Fig. 1A) was collected from Shiping County, Honghe Hani and Yi Autonomous Prefecture, Yunnan Province of China in November 2014. A virus isolate, denoted 14YV855, was obtained by three local lesion transfers on leaves of *C. quinoa*. The virus was maintained on *C. quinoa* and the systemic host *Nicotiana benthamiana* by mechanical inoculation, as described previously (Dong et al. 2008). The virus-infected plants were kept in a temperature-controlled greenhouse set at 25°C with a 16-h photoperiod.

Total RNA was isolated from 14YV855-infected *N. benthamiana* leaves using the GeneMark Plant Total RNA Miniprep Purification Kit (GMBiolab, Taichung, Taiwan) following the manufacturer's instructions. Total RNA was used as a template for one-step real-time reverse-transcription (RT)-PCR using the degenerate primer pair dTospo-F2 (5'-GATCAATCNAARTGGTCDGCWTC-3')/dTospo-R2 (5'-CATDGCACAAGARTGRTAVACWGA-3') to amplify a partial sequence of the L RNA, as described previously (Huang et al. 2018). The PCR products were analyzed by agarose gel electrophoresis and the amplicon was directly sequenced using an ABI 3730XL DNA Analyzer (Mission Biotech Company, Taipei, Taiwan). The nucleotide sequence was analyzed by the Basic Local Alignment Search Tool (BLAST) available at the NCBI (<https://www.ncbi.nlm.nih.gov/>) to determine the virus identity.

Electron microscopy. A Formvar-coated, carbon-stabilized copper grid (300 mesh) was floated for 3 min on one drop of sap extracted from diseased leaf tissues, then dried using a wedge of filter paper and stained for 2 min with 1% (wt/vol) ammonium molybdate in 0.2 M phosphate-buffered saline (pH 7.2). Samples were observed by transmission electron microscopy (TEM) at 80 kV accelerating voltage.

Immunoblotting. Western blotting was performed to detect the N protein of 14YV855 in the infected plants. Total soluble proteins were extracted by grinding 500 mg of plant tissue in 4× protein sample buffer (3% sodium dodecyl sulfate [SDS], 30% glycerol, 0.01% bromophenol blue, and 15% β-mercaptoethanol), followed by incubation at 100°C for 5 min. The supernatant was collected after centrifugation at 8,000 rpm for 3 min. Proteins were resolved by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to polyvinylidene difluoride membranes (PerkinElmer). Membranes were incubated with the monoclonal antibody (MAb) targeted

Fig. 1. Symptomatology, morphology, serology, and identification of 14YV855. **A**, Symptoms displayed on a leaf of a diseased chilli pepper plant. **B**, Virus particles were observed in the leaf tissue of the naturally infected chilli pepper sample collected in Shiping County, Honghe Hani and Yi Autonomous Prefecture, Yunnan Province of China (upper panel) and the *Nicotiana benthamiana* plant mechanically inoculated with 14YV855 (lower panel). **C**, Serological response of 14YV855 reacted with the monoclonal antibody (MAb) against the nucleocapsid protein (NP) of watermelon silver mottle virus (WSMoV). The crude *N. benthamiana* leaf saps infected with other orthospoviruses, including WSMoV, watermelon bud necrosis virus (WBNV), groundnut bud necrosis virus (GBNV), capsicum chlorosis virus (CaCV), calla lily chlorotic spot virus (CCSV), melon yellow spot virus (MYSV), and iris yellow spot virus (IYSV), were used for comparison. The crude sap of healthy *N. benthamiana* (H) was used as negative control. **D**, Primer pair 855M-4060/855M-4588c was used to differentiate 14YV855 from WSMoV, WBNV, GBNV, and CaCV that are indistinguishable by MAb WSMoV-NP, in reverse-transcription PCR. Total RNAs extracted from 14YV855-infected pepper (14YV855-P) and tomato (14YV855-T) were used for the assay. Total RNAs of *N. benthamiana* leaf tissues infected with WSMoV, WBNV, GBNV, and CaCV were used for comparison. Total RNAs of a healthy pepper (H-P) and tomato (H-T) were used as the negative controls.

Table 1. Host range test of 14YV855 by mechanical inoculation^a

Family	Species (variety)	Inoculated leaf	Systemic leaf
Amaranthaceae	<i>Chenopodium quinoa</i>	NS	–
	<i>C. murale</i>	CL	–
Asteraceae	<i>Lactuca sativa</i>	NS	–
	<i>Brassica rapa</i> L. subsp. <i>chinensis</i> group	C	–
	<i>B. campestris</i> L.	–	–
Cucurbitaceae	<i>Cucumis melo</i>	CL	–
Fabaceae	<i>Vigna radiata</i> L. Wilczek	NS	NS, VN
Solanaceae	<i>Capsicum annuum</i> L.	NS	CL
	<i>C. annuum</i> (0311733)	NS	AN, C
	<i>C. chinense</i> (Jacquin PI-152225)	NS	NS, VN
	<i>Nicotiana benthamiana</i>	CL	NS
	<i>N. rustica</i>	NS	NS
	<i>N. tabacum</i> (K326)	NS	NS, VN
	<i>N. tabacum</i> (NC102)	NS	AN, VN
	<i>N. tabacum</i> (Yunyan87)	NS	NS, VN
	<i>Solanum lycopersicum</i>	NS	AN, NS

^a AN, apical necrosis; C, chlorosis; CL, chlorotic lesions; NS, necrotic spots; VN, vein necrosis; and –, no symptoms.

to the WSMoV N protein (MAb WSMoV-NP) at a dilution rate of 1:10,000 (Lin et al. 2005), and capable of detecting capsicum chlorosis virus (CaCV), groundnut bud necrosis virus (GBNV), watermelon bud necrosis virus (WBNV), and WSMoV (Chen et al. 2010). HRP-conjugated goat antimouse immunoglobulin G (Jackson ImmunoResearch) was used as the secondary antibody. The chemiluminescence signals of the target proteins were visualized by a Western Lightning Plus-ECL Kit (PerkinElmer) and recorded using a Multigel-21 image system (Top Bio, Taipei, Taiwan). The leaf tissues of *C. quinoa* separately infected with CaCV, calla lily chlorotic spot virus (CCSV), GBNV, IYSV, melon yellow spot virus (MYSV), WBNV, and WSMoV were analyzed comparatively. The PageRuler Prestained Protein Ladder (Thermo Scientific, Rockford, IL, U.S.A.) was used as a size standard in SDS-PAGE and immunoblotting to determine the molecular weight of the viral proteins.

Host range. Crude sap of *N. benthamiana* systemically infected with 14YV855 was used to mechanically inoculate 13 plant species (Table 1). Each plant species was analyzed in at least two independent experiments, each with three to six replicates. All inoculated plants were kept in a temperature-controlled greenhouse set at 25°C for 4 weeks to observe symptom development. Leaves of the tested plants were confirmed for virus infection by RT-PCR and TEM. A healthy plant was used as a negative control.

Viral genome sequencing and sequence analysis. Leaves of 14YV855-infected *C. quinoa* were harvested at 3 days postinoculation (dpi), and the total RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. A complementary DNA (cDNA) library was synthesized using TruSeq RNA Library Prep Kit (Illumina, San Diego, CA, U.S.A.) following the manufacturer's protocols. Sequencing was performed with an Illumina MiSeq v3 300PE apparatus (Illumina). Fastq reads were qualified using the NGS QC Toolkit (Patel and Jain 2012). The filtered fastq reads were assembled by Platanus, version 1.2.4, with k-mer 127 (Kajitani et al. 2014). Furthermore, primers (Supplementary Table S1) designed from the previously determined sequences were used to amplify the S-, M- and L-RNA genomic segments for Sanger sequencing. The cDNAs were synthesized using the SuperScript III reverse transcription (Invitrogen) according to the manufacturer's instructions. PCR amplifications were performed using the KAPA HiFi DNA polymerase (Kapa Biosystems, Boston, MA, U.S.A.). The reaction was carried out under the following conditions: denaturation at 95°C for 3 min; 30 cycles of 98°C for 20 s, 55°C for 20 s, and 72°C for 60 s; and a final extension of 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 0.8% agarose gel, purified by the GeneMark Plus DNA Clean-Up/Gel extraction kit (GMBiolab, Taichung, Taiwan), then cloned into the TOPO-blunt vector (Invitrogen). Two

Table 2. Nucleotide (nt) and amino acid (aa) sequence identities (%) of the individual coding sequences of 14YV855 to those of other orthotospoviruses

Virus ^a	N		NSs		NSm		Gn/Gc		RdRp	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
CTNRV	90.6	95.3	90.2	93.9	88.0	92.2	87.9	94.1	...	...
WSMoV-TW	83.5	89.9	81.4	85.5	78.5	79.8	79.6	85.9	83.1	92.8
WSMoV-YN	83.8	89.1	81.7	85.7	...	...	...	...	...	...
WSMoV-JP	84.1	89.1	81.8	86.0	...	...	...	...	...	...
WSMoV-GZ	83.3	89.1	80.9	85.7	78.5	79.2	79.5	86.5	83.8	93.1
GBNV	82.5	87.0	81.4	87.4	82.0	87.6	82.0	90.6	81.3	92.0
CaCV	81.3	85.9	77.2	83.1	80.6	86.3	78.5	85.4	80.3	90.1
WBNV	80.6	84.8	79.7	85.2	78.9	82.1	80.2	86.6	80.8	90.8
MVBaV	71.0	73.6	71.6	71.2	75.3	80.5	75.5	81.9	77.2	85.8
TNSaV	64.5	66.5	65.4	63.8	70.0	73.6	69.8	73.0	72.4	77.6
CCSV	64.6	65.1	64.4	64.5	69.6	71.0	69.6	73.3	72.0	77.1
TZSV	64.7	64.5	65.3	63.1	71.4	74.3	70.3	73.1	72.0	76.2
MYSV	63.5	58.3	58.2	48.8	65.6	63.2	65.7	64.6	71.9	75.9
PCSV	61.6	58.3	57.0	46.8	69.4	66.3	67.0	65.4	70.7	73.1
TNRV	60.7	56.5	56.3	48.9	68.7	68.6	65.3	64.6	-	-
PolRSV	54.3	44.3	54.3	48.6	68.0	65.2	64.8	60.5	66.5	67.9
HCRV	55.1	43.9	55.5	48.8	68.7	64.3	64.7	61.8	67.4	68.1
TYRV	54.5	43.9	55.6	51.0	69.2	67.2	65.3	64.2	67.2	67.8
IYSV	56.2	45.2	54.8	50.5	69.1	68.5	64.7	63.3	67.0	69.4
SVNV	44.3	33.1	39.7	17.2	51.0	33.8	51.5	30.5	54.5	44.1
MeSMV	47.1	32.2	38.2	16.9	55.7	39.2	51.9	32.3	46.8	45.4
GRSV	46.5	31.8	37.1	16.1	52.7	38.4	48.6	35.0	54.9	46.6
TSWV	47.1	31.0	37.9	14.0	50.3	37.2	48.8	34.9	55.2	45.0
PNSV	46.5	30.2	38.3	16.8	...	...	...	...	...	...
ZLCV	48.2	29.6	24.2	16.1	51.0	34.1	48.4	35.4	46.2	45.9
ANSV	44.7	29.4	...	...	...	...	...	...	...	...
CSNV	46.3	29.2	37.2	16.8	52.9	39.1	48.2	35.1	55.4	45.9
BeNMV	43.7	28.6	40.7	17.2	53.7	36.8	51.9	32.0	54.5	43.8
INSV	45.1	28.5	38.6	17.8	51.7	39.4	49.8	35.6	55.6	46.2
TCSV	46.4	28.2	...	...	51.8	39.1	47.8	35.2	46.8	45.4
GCFSV	41.3	19.7	37.7	17.5	48.7	36.7	46.9	29.8	50.7	36.6
GYSV	41.2	19.3	37.8	13.0	...	...	...	...	...	...
LNRV	37.9	19.1	37.9	20.7	...	...	...	...	...	...

^a Names and abbreviations: alstroemeria necrotic streak virus (ANSV), bean necrotic mosaic virus (BeNMV), calla lily chlorotic spot virus (CCSV), capsicum chlorosis virus (CaCV 2), cherry tomato necrotic ringspot virus (CTNRV), chrysanthemum stem necrosis virus (CSNV), groundnut chlorotic fan-spot virus (GCFSV), groundnut bud necrosis virus (GBNV), groundnut ringspot virus (GRSV), groundnut yellow spot virus (GYSV), hippeastrum chlorotic ringspot virus (HCRV), impatiens necrotic spot virus (INSV), iris yellow spot virus (IYSV), lisianthus necrotic ringspot virus (LNRV), mulberry vein banding associated virus (MVBaV), melon yellow spot virus (MYSV), melon severe mosaic virus (MeSMV), pepper chlorotic spot virus (PCSV), pepper necrotic spot virus (PNSV), polygonum ringspot virus (PolRSV), soybean vein necrosis virus (SVNV), tomato chlorotic spot virus (TCSV), tomato necrotic ringspot virus (TNRV), tomato necrotic spot-associated virus (TNSaV), tomato spotted wilt virus (TSWV), tomato yellow ring virus (TYRV), tomato zonate spot virus (TZSV), watermelon bud necrosis virus (WBNV), and watermelon silver mottle virus (WSMoV).

recombinants were sequenced. The obtained nucleotide sequences were assembled and analyzed with the aid of DNAMAN, version 5.0 (Lynnon Biosoft). Phylogenetic trees were constructed using the neighbor-joining method with 1,000 bootstrap replications in

MEGA 6.0 (Tamura et al. 2013). The sequences of likely parental isolates (WSMoV, GBNV, WBNV, CaCV, and TZSV) were examined with recombination detection methods implemented in the software Recombination Detection Program, version 4 (RDP4), including

Fig. 2. Phylogenetic trees of orthotospoviruses based on the amino acid sequences of **A**, N protein; **B**, NSs protein; **C**, NSm protein; **D**, Gn/Gc protein; and **E**, RNA-dependent RNA polymerase (RdRp). Bootstrap values on the branches represent the percentage of 1,000 bootstrap replicates. Virus 14YV855, named chilli yellow ringspot virus (CYRSV), studied in this research is indicated by arrows. Abbreviations of orthotospovirus names are provided in Supplementary Table S2.

Table 3. Summary of unique recombination events identified by the Recombination Detection Program (RDP), version 4 (RDP4)^a

ORF	Event	Breakpoint position in recombinant sequence			Parental sequences ^b	
		Begin	End	Recom	Minor	Major
L	1	8,033	8,187	CYRSV	WSMoV-TW	Unknown (WSMoV-GZ)
	2	618	707	CYRSV	CaCV	WSMoV-GZ
	3	850	1,141	WBNV	Unknown (CaCV)	WSMoV-TW
NSm	1	88	324	GBNV	Unknown (CaCV)	CYRSV
N	1	140	334	WBNV	WSMoV-GZ	Unknown (CYRSV)
	2	655	86	WBNV	WSMoV-GZ	CaCV

(Continued on next page)

^a ORF = open reading frame and Recom = recombinant.

^b The sequence listed as unknown was used to infer the existence of a missing parental sequence.

^c NS = no significant *P* value was recorded for this recombination event using this method.

RDP, GENECONV, Bootscan, Maxchi, Chimaera, SiScan, PhylPro, LARD, and 3Seq (Martin et al. 2015), to identify the putative recombination and recombination breakpoints using default settings. The orthotospoviral sequences used for comparison and obtained from the GenBank database are listed in Supplementary Table S2.

Specific primers and detection of 14YV855 from field samples.

The genomic sequences of 14YV855 were compared with those of CaCV, GBNV, WBNV, and WSMoV using the ClustalW program (Larkin et al. 2007) to determine the divergent sequence segments. The alignment file was used for designing primers specific to 14YV855. RT-PCR was conducted using the GeneMark One-step RT-PCR kit (GMBiolab) according to the manufacturer's instructions. The reaction mixture consisted of 1 µg of total RNA, 200 nM individual primers, 25 U of one-step RT-PCR enzyme mix, 5 µl of reaction buffer, and 5 µl of enhancer buffer in a 25-µl volume. RT was carried out at 50°C for 30 min, and PCR was performed for 35 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 1 min, and an additional final reaction at 72°C for 7 min. The PCR products were visualized by 1% agarose gel electrophoresis using M5 HiPure Gelred (Mei5biao, Beijing, China) as the nucleic acid stain.

The newly designed assay was used to screen 46 plant samples with virus-like symptoms that were collected from August 2017 to October 2018 from 51 fields where 14YV855 was originally collected. They include 26 chilli plants, 10 tomato samples (six fruit and four leaves), five wax gourd leaves, four zucchini leaves, and one cucumber leaf. In addition, nine tomato fruit showing typical orthotospovirus symptoms were collected from Yuanmou County, Chuxiong Yi Autonomous Prefecture, Yunnan Province in January 2019 and similarly tested.

Results

Identification of 14YV855 as an orthotospovirus serologically related to WSMoV. The orthotospovirus-like enveloped spherical particles were observed in the leaf tissue of the diseased chilli pepper sample naturally infected with 14YV855 and the *N. benthamiana* plant mechanically inoculated with the virus (Fig. 1B). A positive result was observed in immunoblot analysis of a crude sample of 14YV855 using the MAbs WSMoV-NP. A protein of about 31 kDa, presumably the N protein of 14YV855, similar to that CaCV, GBNV, WBNV, and WSMoV, was detected (Fig. 1C). Using the degenerate primer pair dTospo-F2/dTospo-R2, an approximately 300-bp DNA fragment corresponding to the conserved region of orthotospoviral L RNA was amplified from the 14YV855-infected *C. quinoa* leaves (data not shown). The BLAST analysis indicated that the nucleotide sequence of the directly sequenced amplicon shared the highest identity of 87 to 88% with the RdRp gene of GBNV.

Host range and symptomatology of 14YV855. Isolate 14YV855 was inoculated mechanically onto 13 distinct plant species, including two varieties of *Capsicum annuum* and three varieties of *N. tabacum*, to assess host range and symptomatology. As expected, all of the solanaceous crops, including TSWV-resistant pepper (*C. chinense*

Jacquin PI-152225 line carrying the *Tsw* gene), exhibited systemic symptoms postinoculation (Table 1). Apical necrosis, chlorosis, chlorotic lesions, necrotic spots, and vein necrosis were observed in these solanaceous crops. *Vigna radiata* (L.) R. Wilczek of the family Fabaceae exhibited systemic necrotic spots and vein necrosis symptoms. *Brassica rapa* L. subsp. *chinensis* group of the family Brassicaceae exhibited chlorosis on the inoculated leaves but no symptoms were observed on *B. campestris*. Local symptoms were seen on the inoculated leaves of *Chenopodium quinoa*, *C. murale*, *Lactuca sativa*, and *Cucumis melo*. RT-PCR with the primer pair specific to 14YV855, 855M-4060 (5'-GTTTCTTATCAACTCCAACAC-3')/855M-4588c (5'-TATCGAAGAAGACGAAGTTTA-3'), was designed and used to confirm the 14YV855 infection in the inoculated plants (Fig. 1D).

Analysis of the whole genome sequence of 14YV855. To obtain the complete genome sequences of 14YV855, the infected leaves of *Chenopodium quinoa* were processed by cDNA synthesis and high-throughput sequencing with an Illumina MiSeq v3 300PE system. In total, 5,424,494 reads (20 to 24 nucleotides [nt]) with quality Q30 > 91% were generated, and 6,456 unique reads were mapped to genome sequences of orthotospoviruses. However, the obtained sequences did not cover the whole genome of 14YV855. Thus, specific primers designed from the RNA-sequencing sequences were used to obtain the whole-genome sequences of 14YV855 using conventional RT-PCR, cloning, and Sanger sequencing.

The complete sequence of 14YV855 S-RNA (GenBank accession number MH779495) is 3,428 nt, containing the NSs and N ORFs separated by a 1,147-nt AU-rich IGR. The NSs ORF is 1,320 nt long at the position nucleotides 67 to 1,386 of the *v*-sense RNA and encodes a 439-amino-acid (aa) (49.8-kDa) protein. The N ORF is 828 nt long at the position nucleotides 3,361 to 2,534 of the *vc* RNA strand and encodes a 275-aa (30.5-kDa) protein. The 14YV855 M-RNA (MH779496) was determined to be 4,781 nt long, containing NSm and Gn/Gc ORFs, separated by a 389-nt AU-rich IGR. The NSm ORF encodes a 307-aa (34.1-kDa) protein from the position nucleotides 56 to 979 of the *v* RNA. The Gn/Gc ORF encodes a 1,121-aa (127.3-kDa) Gn/Gc precursor protein from the position nucleotides 4,734 to 1,369 of the *vc* RNA. The 14YV855 L-RNA is 8,917 nt in length (MH779497), containing one large ORF at the position nucleotides 33 to 8,669 of the *vc* RNA strand which encodes a 2,878-aa (331.4-kDa) RdRp protein.

The coding sequences of 14YV855 were compared with those of the 29 characterized official and tentative *Orthotospovirus* spp. (Table 2). The N protein of 14YV855 sharing less than 90% aa identity with that of the analyzed orthotospoviruses (29.6 to 89.9%) suggests that 14YV855 is a distinct orthotospovirus, provisionally named as chilli yellow ringspot virus (CYRSV). We also found that the N, NSs, NSm, and Gn/Gc proteins of CYRSV have greater than 90% aa identities with those of an unpublished orthotospovirus, named cherry tomato necrotic ringspot virus (CTNRV), whose S- and M-RNA sequences were recently submitted to the GenBank (MG656997 and MG656998) (Table 2), indicating that CYRSV and CTNRV are members of the same species.

Table 3. (Continued from previous page)

Score for the detection methods in RDP4 ^c								
RDP	GENECONV	Bootscan	Maxchi	Chimaera	SiScan	PhylPro	LARD	3Seq
1.52E-02	NS	4.69E-02	NS	NS	3.84E-02	NS	NS	3.19E-02
2.56E-02	NS	NS	NS	NS	NS	NS	NS	NS
3.44E-02	NS	NS	NS	NS	NS	NS	NS	NS
4.10E-02	NS	NS	3.74E-03	1.23E-02	NS	NS	NS	NS
2.04E-02	NS	NS	3.11E-02	6.70E-03	NS	NS	NS	3.07E-02
1.69E-02	NS	NS	NS	4.15E-02	NS	NS	NS	NS

Sequence analyses revealed that the NSs, NSm, and Gn/Gc proteins of CYRSV shared the highest amino acid identities—87.4, 87.6, and 90.6%, respectively—with those of GBNV but the N and RdRp proteins shared the highest amino acid identities—89.9 and 93.1%, respectively—with those of WSMoV. Phylogenetic analyses demonstrated that the N and RdRp proteins of CYRSV are the most related to WSMoV, while the other proteins are closely related to GBNV (Fig. 2). The results of sequence and phylogenetic analyses suggested the occurrence of recombination among CYRSV, CaCV, GBNV, WBNV, and WSMoV. Recombination was examined by RDP4. Two putative recombination events within the L RNA of CYRSV were detected, with CaCV and WSMoV as the potential parental sequences. Recombination was also detected within the L RNA of WBNV and the potential parental sequences were isolates of WSMoV. A putative recombination event between CYRSV and GBNV was detected in the NSm ORF of the M RNA. Additionally, two putative recombination events were detected within the N ORF of WBNV S RNA that involved CaCV, WSMoV, and CYRSV as the potential parental sequences (Table 3).

RT-PCR for identification and detection of CYRSV. The divergent sequences between the genomic sequences of CYRSV and four serologically related orthotospoviruses (CaCV, GBNV, WBNV, and WSMoV) were used for designing primers specific to CYRSV. The primer pair 855M-4060/855M-4588c designed from the M RNA sequence of 14YV855 was successfully used to differentiate CYRSV from CaCV, GBNV, WBNV, and WSMoV in RT-PCR. A 0.5-kb specific PCR product from the total RNA of CYRSV-infected plant tissues was amplified (Fig. 1D) and the amplicons were sequenced to verify their specificity (data not shown).

In chilli pepper- and tomato-growing fields of Shiping, Honghe of Yunnan Province, approximately 2% of plants showing yellow ring-spots, necrotic spots, and mottling on leaves were observed. In tomato-growing fields of Yuanmou, Chuxiong of Yunnan Province, the percentage of diseased plants showing typical orthotospoviral symptoms varied between 5 and 20% depending on tomato varieties and growth stage, with an average of 12%.

RT-PCR analysis of the field-collected samples showed CYRSV infections in 3 of 26 chilli pepper samples and 2 of 10 tomato samples

that were collected from the field in Shiping in which CYRSV was initially found (Table 4; Fig. 3). No other orthotospoviruses were detected from the Shiping's samples with the degenerate primer pair dTospo-F2/dTospo-R2. The CYRSV infections were also detected in three of nine tomato fruit from Yuanmou (Table 4). TSWV was detected from the other five symptomatic tomato fruit samples from Yuanmou (data not shown). No mixed infections of orthotospoviruses were found in these samples. The specificities of obtained RT-PCR fragments were verified by cloning and sequencing. The amplicons had a high nucleotide identity between 90.0 to 100% with CYRSV (Supplementary Table S3). No orthotospoviruses were detected in the tested cucurbit samples.

Discussion

In the present study, the virus isolate 14YV855, later named CYRSV, was characterized as a putative new *Orthotospovirus* sp. Orthotospovirus diseases have become the main threat that constraints the yield and quality of economically important crops, including tomato, pepper, and tobacco crops, in China. To date, at least 11 additional orthotospoviruses—CCSV (Liu et al. 2012), CaCV (Chen et al. 2007), GYSV (Kang et al. 2014), HCRV (Dong et al. 2013), impatiens necrotic spot virus (Cheng et al. 2010), MVBaV (Meng et al. 2015), pepper chlorotic spot virus (PCSV) (Zheng et al. 2017), TNSaV (Yin et al. 2014; Zheng et al. 2016), TSWV (Dong et al. 2010), TZSV (Dong et al. 2008), and WSMoV (Rao et al. 2013)—have been reported in China. Most of these viruses, including recently characterized species HCRV, PCSV, TNSaV, and TZSV, were reported from Yunnan Province. TSWV is the most important orthotospovirus distributed all over Yunnan Province due to the spread of *Frankliniella occidentalis*.

CYRSV, as well as some orthotospoviruses such as TSWV, TZSV, TNSaV, PCSV, and CYRSV, caused similar symptoms of chlorotic ringspots and necrosis on leaves and fruit of the diseased chilli pepper and tomato plants. Polyclonal antisera to the N protein are conventionally used to detect orthotospoviruses. However, serological cross-reaction among the members of a serogroup makes identifying virus species with serology insufficient (Chen et al. 2010; Huang et al. 2018). Although MABs are produced to facilitate the identification of orthotospoviruses (Chen et al. 2010; Chen et al. 2016), some viruses such as CaCV, GBNV, WBNV, and WSMoV remain serologically indistinguishable. An efficient approach for orthotospovirus identification is the use of RT-PCR with genus-universal degenerate primers coupled with sequencing and BLAST analysis of obtained sequences (Chen et al. 2012; Chu et al. 2001; Cortez et al. 2001; Huang et al. 2018). The integrity of the viral genome sequences available in GenBank contributes to the correctness of virus identification.

CYRSV and WSMoV share the highest 89.9% identity, very close to 90%, of the N protein with each other, similar to that of PCSV and tomato necrotic ringspot virus (Cheng et al. 2014; Hassani-Mehraban et al. 2011). To avoid argument, the divergence of the other viral proteins such as NSs and NSm proteins can be references to demarcate an *Orthotospovirus* sp. (Cheng et al. 2014; Zheng et al. 2017). In this case, the NSm protein of CYRSV sharing a significantly low amino

Table 4. Detection results of chilli yellow ringspot virus (CYRSV) in Shiping and Yuanmou Counties of Yunnan Province

Location	Collection year	Crop	Number of tested samples	Number CYRSV positive
Shiping	2017	Chilli pepper	14	1
		Tomato	10	2
		Wax gourd	5	0
		Cucumber	1	0
		Chilli pepper	12	2
	2018	Zucchini	4	0
Yuanmou	2019	Tomato	9	3

Fig. 3. Detection results of partial samples collected from Shiping, Honghe, Yunnan Province by reverse-transcription PCR with the primer pair 855M-4060/855M-4588c. Lane 1, DL2000 DNA Marker (TaKaRa, Dalian, China); lanes 2 and 23, chilli yellow ringspot virus-infected *Nicotiana benthamiana* plants used as the positive control; lane 3, a healthy *N. benthamiana* plant used as the negative control; lane 4, wax gourd collected in 2017; lanes 5 to 8, zucchini collected in 2018; lanes 9 to 15, chilli pepper collected in 2017; lanes 16 to 22, chilli pepper collected in 2018; lane 24, TZSV-infected chilli pepper; and lane 25, tomato spotted wilt virus-infected chilli pepper. Expected amplicons (529 bp) are indicated by an arrow.

acid identity of 79.2 to 79.8% with multiple WSMoV isolates (Table 2) can demonstrate the distinction between CYRSV and WSMoV.

CTNRV is another member of the new *Orthotospovirus* sp. to which CYRSV belongs. Both CYRSV and CTNRV were collected in Yunnan Province but the collection years were different. CYRSV was discovered in 2014 whereas CTNRV was found 2 years later. Afterward, more CYRSV-related virus isolates were also detected in Yunnan Province in 2017 to 2019. Undoubtedly, CYRSV is the type isolate for this new *Orthotospovirus* sp. because it is fully characterized biologically and molecularly compared with CTNRV. Although the incidence of orthotospoviral diseases was low (approximately 2%) in Shiping County where CYRSV was first collected, the average disease incidence of orthotospoviruses was about 12% (up to 90% in parts of fields) in Yuanmou County. The orthotospovirus-transmitting vectors *F. occidentalis* and *Thrips palmi* are dominant species in Yunnan Province. Thus, it is necessary to explore the insect vector and distribution of CYRSV in Yunnan Province in the future for control and prevention of the virus.

Both reassortment and recombination have been demonstrated to contribute to the molecular diversity of the genus *Orthotospovirus*. The reassortment and recombination have been shown to be an important evolutionary mechanism among different TSWV isolates (Lian et al. 2013; Qiu et al. 1998; Tentchev et al. 2011; Zhang et al. 2016). The reassortment of different orthotospoviruses was also reported in previous studies (Silva et al. 2019; Webster et al. 2011). Results of the studies showed that the M-RNA segments are frequently exchanged between groundnut ringspot virus and tomato chlorotic spot virus, which have a close phylogenetic relationship and indistinguishable serology. Recombination events can be found within the L, NSm, and N ORFs among CYRSV, CaCV, GBNV, WBNV, and WSMoV, and CaCV, GBNV, and WSMoV may be the genetic parents of CYRSV. To our knowledge, chili pepper and tomato are the common natural hosts of CaCV, GBNV, WBNV, and WSMoV (Kunkalikar et al. 2011; Yin et al. 2016), and CYRSV can also infect both crops. This implies that the possibility of recombination coupled with mutations might occur within a common host that is coinfecting with these orthotospoviruses. GBNV and WBNV have not yet been reported in China and, hence, their presence needs to be explored. High levels of recombination events can be detected in the genome of different TSWV isolates worldwide, suggesting that recombination, as well as reassortment, might be an important evolutionary mechanism in orthotospoviruses (Zhang et al. 2016). Further studies are needed to determine the possible contribution of recombination to the evolution of new *Orthotospovirus* spp.

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