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Research Article
Investigation of *Tomato chlorosis virus* Infection in Tomato Growth Facilities in Shanghai, China

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Abstract

Background and Objective: *Tomato chlorosis virus* (ToCV), which can strongly decrease tomato yields, has been found in farms in multiple provinces and cities across China, but in many cases, the infectious strains causing tomato chlorosis were not reported. This study aimed to investigate the ToCV epidemic situation in tomato growth facilities in Shanghai and clarify the relationship between the locally circulating infectious strains and the published strains. **Materials and Methods:** In this study, random leaf samples were collected from greenhouse-grown tomatoes at 3 different locations in Shanghai in November, 2020. Using ToCV-specific primers, we detected ToCV in 67-95% of samples from these greenhouses by reverse transcription PCR and were able to obtain full-length sequences of ToCV from all 3 locations. **Results:** The sequences of the 3 isolates were found to be highly similar to each other and results from sequence alignment and phylogeny analysis of RNA1 and RNA2 indicate a close relationship between these ToCV isolates and the ones from Korea, with sequence identities above 99.7%. In addition, for one of the greenhouses that grow 3 crops of tomatoes per year, we monitored ToCV infection rates for 2 additional contiguous planting seasons. It was found that the infection rate was the lowest in early summer when whitefly counts were low and regular greenhouse management was ongoing. **Conclusion:** Current study indicates that ToCV infection may be the main reason underlying tomato chlorosis in Shanghai tomato growth facilities and greenhouse management is helpful but not sufficient for disease control.

Key words: *Tomato chlorosis virus*, infection rate, full-length sequence, greenhouse management, whitefly counts

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Viral diseases are a major problem affecting tomato production worldwide[1]. Among these, the *Tomato chlorosis virus* (ToCV) was found in some major tomato-producing areas and caused significant economic losses in many countries including China, which is the largest tomato-producing country in the world. In addition to tomato, ToCV also infects other vegetables, such as peppers and eggplants in the Solanaceae family, apricot in the Rosaceae family and spinach in the Chenopodiaceae family[2,3] and so directly threatens Chinese vegetable production. Thus, it is important to investigate the prevalence of ToCV and its effects on crop production with the future aim of controlling or eradicating the virus from production systems.

Tomato chlorosis disease was first described in 1989 but it was not until 1998 that ToCV was confirmed as the causal pathogen[4]. The ToCV belongs to the genus *Crinivirus* in the Closteroviridae family. It is a single-stranded positive RNA virus with 2 genomic RNA strands, RNA1 and RNA2[5]. The RNA1 contains 8596 nucleotides (nts) for most isolates, which contain 4 Open Reading Frames (ORFs) that encode protein kinase, methyltransferase, helicase and RNA-dependent RNA Polymerases (RDRP), while RNA2 is about 8242 nts long and contains nine ORFs that encode heat shock protein 70 (HSP70), movement protein (MP), coat protein (CP) and other proteins. These proteins are mainly involved in virus cell-to-cell movement and systemic infection and viral particle packaging. However, the biological functions of some of the encoded proteins remain unclear. Sequence analysis of all publically available isolates shows relatively small sequence variation while the HSP70 and RDRP sequences are highly conserved[6]. Hence, HSP70 and RDRP are often used for primer design in the identification of ToCV infection by reverse transcriptional (RT) PCR.

In infected tomato plants, ToCV is generally localized in the phloem cells. The plants develop disease symptoms about 3 weeks after infection. The symptoms start as leaf chlorosis and yellowing at the lower part of the plants which gradually progresses upward. In severe cases, the lower chlorotic leaves exhibit reddish-brown spots and photosynthesis is affected, leading to insufficient fruit development and reduced yield. Furthermore, the disease symptoms can be exacerbated when the plants are co-infected with other viruses[7]. The ToCV is transmitted by whiteflies both in the field and in greenhouses. Thus, the main strategy and method for disease control are to minimize whitefly populations by physical and chemical means, such as setting up insect nets and hanging yellow sheets[8,9]. These methods are shown to provide effective local control to a certain extent but add to the management burden and increase production costs. At present, there are no tomato varieties that are proven to have a specific resistance to ToCV infection.

So far, tomato ToCV disease has been reported in more than 30 countries on all continents except Australia and New Zealand[10]. In China, the disease was first reported in Taiwan in 2004 and then was found in Beijing, Shandong, Shanxi and Jiangsu Provinces in 2010[3,11-13]. The disease appears to be further spreading across China[3], indicating the threat of larger disease outbreaks. Furthermore, tomato plants were found to be infected by both ToCV and *Tomato yellow leaf curl virus* in some provinces which aggravated the disease symptoms[14]. Thus, more epidemic investigations and tracking are becoming more urgent.

Shanghai is not only the economic engine of China but also has a well-developed urban agriculture system. Tomato is one of Shanghai's most important cash crops and is mainly cultivated in growth facilities. Although there have been no official reports of ToCV infection in Shanghai, to our knowledge no studies have yet been conducted. Thus, investigations were carried out to observe the ToCV infection in 3 tomato growth facilities located in different areas of Shanghai and one case, monitored infection rates across 3 consecutive growing seasons.

MATERIALS AND METHODS

Tissue sampling: Young leaves of tomato (*Solanum lycopersicum*) plants were randomly collected in 2 greenhouses in Shanghai, China in November, 2019, after the fruits were mostly harvested and greenhouse management was neglected. The greenhouses GH-SIPPE and GH-SIA are located in Songjiang and Fengxian District (30°56'26.72"N, 121°8'1.33"E and 30°53'40.23"N, 121°23'30.29"E). The greenhouses are mainly used for scientific research by local research institutes. In addition, 3 consecutive samplings were conducted in the greenhouse of Shanghai Normal University GH-SNU (30°50'48.42"N, 121°31'51.85"E). Samples were collected in August, 2019 when fruits were mostly harvested and in November, 2019 when fruits were being harvested and in June, 2020 when the plants were flowering and producing fruits. The leaves were immediately frozen in liquid nitrogen and stored at -80°C.

Total RNA extraction and ToCV detection: The total RNA of tomato leaves was extracted using tri-reagent (SIGMA) following the manufacturers instructions. For 200 mg of tissue, 1 mL of extraction buffer was used. In brief, the leaf

Table 1: All primers for RT-PCR analysis

Primers	Sequences
ToCV-RNA1-F	TACGCATACGGCGACTTTGG
ToCV-RNA1-R	TCTTGGCCCTGTACTGAGAAAC
ToCV-RNA2-F	TCGAAGTCTCGTTTCTCGTAACA
ToCV-RNA2-R	TGTGACCTCCCTCTCGATCT
ToCV1-F	RAAATAGTATTCGTGTGATYACACAAAGTAC
ToCV1-R1	GATGTCTCTCACCATATTCCTCACAATG
ToCV1-F2	TACAGAACTGTTGTTGTTCAAGCCAATGT
ToCV1-R	CGACCTATTTATTTATATACTAGATSTACCAAGGT
ToCV2-F	GAAATACWAGTCCAGGTGTTTCCTGTGGGTA
ToCV2-R	CGACCTATTTATTTATATACTAAATCTACCAAGGTYTACG

tissues were homogenized in liquid nitrogen and mixed well with a proportional volume of extraction buffer. After 5 min of incubation at room temperature, chloroform was added to the extract and the mixture was vortexed. The extraction mixture was kept at room temperature for 5-15 min and centrifuged for 15 min at full speed at 4°C. The water phase supernatant containing total RNA was obtained and mixed with an equal volume of isopropanol followed by incubation on ice for 15 min and centrifugation at 12,000 g for 10 min. The pellet was then washed twice with 75% ethanol, air-dried for 5 min and re-suspended in nuclease-free water for further analysis or storage at -80°C. For full-length cDNA synthesis, the total RNA was treated with DNase (Invitrogen) and further purified. The purified RNA was stored at -80°C.

Based on the conserved sequences of ToCV RNA1 and RNA2 2 pairs of gene-specific primers were designed corresponding to the methyltransferase domain and HSP70 genes, respectively (ToCV-RNA1/2-F/R) in Table 1. The cDNA was made using 1-2 µg of total RNA per sample, ToCV specific primers (ToCV-RNA1-R and ToCV-RNA2-R) and MMLV reverse transcriptase (Takara), according to the protocol provided by the manufacturer. The resulting products were then used as a template in the following PCR reaction. The PCR reaction mix was as follows: 10 µL2×Hieff® Taq DNA polymerase Robust PCR Master Mix (YEASEN Bio), 1 µL forward (F) primer (ToCV-RNA1-F or ToCV-RNA2-F), 1 µL reverse (R) primer (ToCV-RNA1-R or ToCV-RNA2-R), 1 µL cDNA template and 7 µL nuclease-free water to make up the volume to 20 µL. The PCR program used was as follows: 94°C, 5 min, 94°C, 30 sec, 58°C, 20 sec, 72°C, 30 sec, 30 cycles 72°C, 5 min. The PCR products were detected by 1% agarose gel electrophoresis and the bands of expected sizes were purified with the AXYGEN DNA gel recovery kit (Corning life sciences) and sent to Shanghai Shenggong Bioengineering Company for sequencing.

Sequence analysis: Due to the high similarity of the virus sequences among various isolates, four pairs of primers were used to obtain the full-length cDNA sequences of ToCV RNA1 and RNA2 by RT-PCR. The RT was performed using 5-10 µg of total RNA from the infected samples identified above and SuperScript III reverse transcriptase (Invitrogen) was used according to the manual. Phusion ultra-fidelity DNA polymerase (NEB) was used to amplify the full-length cDNA and the PCR products were purified from the agarose gel and sequenced by Shenggong Bioengineering Company. The PCR reaction system consisted of 10 µL 5×Phusion GC buffer, 1µLdNTPs, 2.5 µL 10 mM F primer, 2.5 µL 10 mM R primer (ToCV1-F/ToCV-RNA1-R, ToCV-RNA1-F/ToCV1-R1, ToCV1-F2/ToCV1-R, ToCV2-F/ToCV2-R), 1 µL cDNA template, 1.5 µL DMSO, 0.5 µL Phusion ultra-fidelity DNA polymerase, 1 µL RNase H and additional ultrapure water to make up the volume to 50 µL. The following PCR program was used: 98°C, 2 min, 98°C, 15 sec, 65°C, 30 sec, 72°C, 150 sec for RNA1 fragment 1 or 235 sec for RNA1 fragment 2 or 340 sec for RNA1 and RNA2 full-length cDNA, 72°C, 10 min.

In addition, to further confirm the sequences, multiple primer pairs in Table 1 were used to further amplify several cDNA fragments which were cloned into a pJET blunt-end cloning vector (Thermo scientific) and multiple (2-3) clones for each fragment were sequenced and all sequences were compared. Finally, the full-length sequences were compared with the 24 RNA1 sequences and 26 RNA2 sequences from 9 different countries available in the NCBI database using Clustal X for sequence alignment and the maximum likelihood method was used to obtain a phylogenetic tree in MEGA7[15].

RESULTS

Most tomato plants harvested were infected by ToCV: To investigate the virus strains infecting the tomato plants grown in local growth facilities, sampled leaf tissues of most plants showed severe disease symptoms at the end of the growing season. The most common symptom was yellowish leaves while some plants had curled leaves or whitish lesions in Fig. 1b, c and e but yet a small portion of plants appeared normal and were randomly distributed in the greenhouses in Fig. 1a and d. At that time, greenhouse management was neglected because fruit harvesting was nearly completed in 2 greenhouses, GH-SIPPE and GH-SIA. A large number of whiteflies were found during sampling in these 2 greenhouses. In the GH-SNU greenhouse in November 2019, regular pesticide spray and fertilization were ongoing and fruits were being harvested but many whiteflies were found. More than half of the plants exhibited disease-like symptoms.

Upper panels in both Fig. 2a and b showed the results for RNA1 detection and the lower panels for RNA 2 detection, M: 2000 bp marker, N: Negative control, P: Positive control, 1-12 or 16, individual tomato samples and red arrows indicate the expected PCR products from ToCV infected tomato plants.

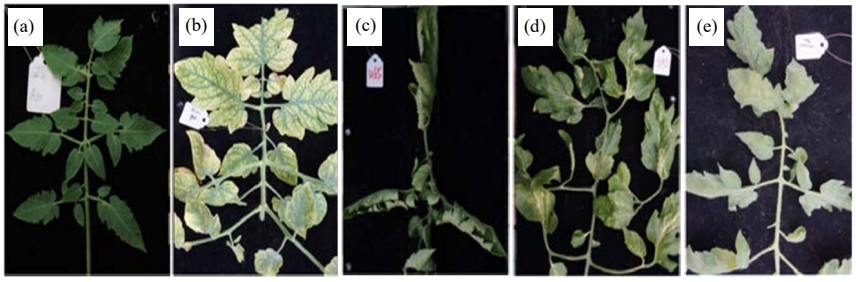

Fig. 1(a-e): Symptomatic leaves in the greenhouse, (a) Leaves that appeared normal, (b) Yellowish, (c) Curled, (d) Necrotic and (e) Leaf showing whitish lesions

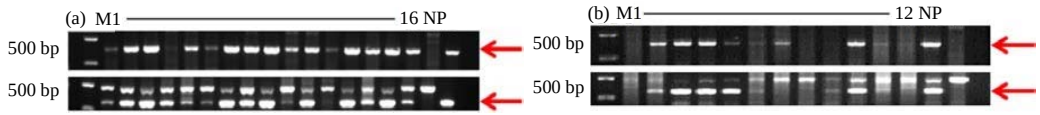

Fig. 2(a-b): RT-PCR results of ToCV and RNA templates were extracted from the tissues harvested, (a) GH-SIA and (b) GH-SNU

In all 3 greenhouses studied more than 1000 tomato plants per house were being grown. We harvested young leaves from 48, 45 and 27 random plants in November, 2019. During sampling, the morphological features of each plant were recorded. To figure out the infection rate of all harvested samples in each greenhouse, specific primers for amplifying the conserved genomic sequences of ToCV were used for RT-PCR analysis. The expected PCR products from ToCV infected tissues were 385 bp (RNA1, top panels) in Fig. 2a and b and 321 bp (RNA2, bottom panels) in Fig. 2a and b. The samples that did not generate PCR products of the right sizes (PCR-negative samples) were considered non-infected (Fig. 2a and b). Sent 5% of the PCR products with the right sizes for sequencing and they all showed 99.4-100% identity to the known ToCV sequences with lower similarity to the diverged China-Taiwan isolate. Thus, all the samples were analyzed by their RT-PCR products from RNA1 and RNA2. The results showed that ToCV cDNA was detected in 92.1, 94.9 and 67.2% of the samples from GH-SIPPE, GH-SIA and GH-SNU, respectively in Table 2. Most (89.5-100%) of the positively testing samples came from plants that showed yellowish leaves (Fig. 1b).

Infection rate of ToCV was lower in late spring: To understand the impact of large-scale infection in the greenhouse on the next round of tomato planting, a continuous follow-up survey was conducted in the GH-SNU greenhouse, where tomato planting is usually rotated 3 times a year. The growing seasons are September-December, February-May and June-August. However, due to the COVID-19 pandemic in 2020, the spring growing rotation was 1 month delayed and was from the end of March, to the beginning of July. The 1st sampling was done at the end of August, 2019 after fruit harvesting was mostly completed and the 2nd sampling was carried out in November, 2019 when the fruits were ripening and some were being harvested. The third sampling occurred in June, 2020 when the plants were flowering and bearing fruit and the plants looked normal and healthy and the whitefly population was low. The virus detection analysis revealed that 67.2, 62.9 and 7.1% of the tissues from the above 3 batches of samples were infected with ToCV, respectively in Table 2. For the samples harvested in the 3rd sampling, of those that tested positive for ToCV only 33.3% were from plants with yellowish leaves, indicating early-stage infection. The ToCV genome sequences across Shanghai isolates were highly similar based on the sequencing results of the full-length cDNA of ToCV RNA1 and RNA2 by RT-PCR using the total RNA from positively infected plants from the 3 greenhouses. The Shanghai ToCV isolates, ToCV-SNU (GenBank accession numbers MW490607 and MW490608), ToCV-SIPPE (MW490609 and MW490610) and ToCV-SIA (MW490611 and MW490612) were all submitted to NCBI. Results from sequence comparison analysis indicate that their RNA1 sequences were highly similar, with only 4-8 nts being polymorphic. Due to these variations, 2 Amino Acids (AA) at positions 258 and 299 of papain exhibit diversity while most of the other variations were synonymous mutations. The sequence similarity across 3 locations for RNA2 is 99.7% and P4, P8, HSP70, P59, P27 and minor CP all show 1-4AA differences. Nevertheless, the RNA2 of ToCV-SNU and ToCV-SIPPE was 99.9% identical.

In addition, these sequences were compared with publicly available sequences of isolates from other countries using cluster analysis. The phylogeny trees of RNA1 in Fig. 3a and

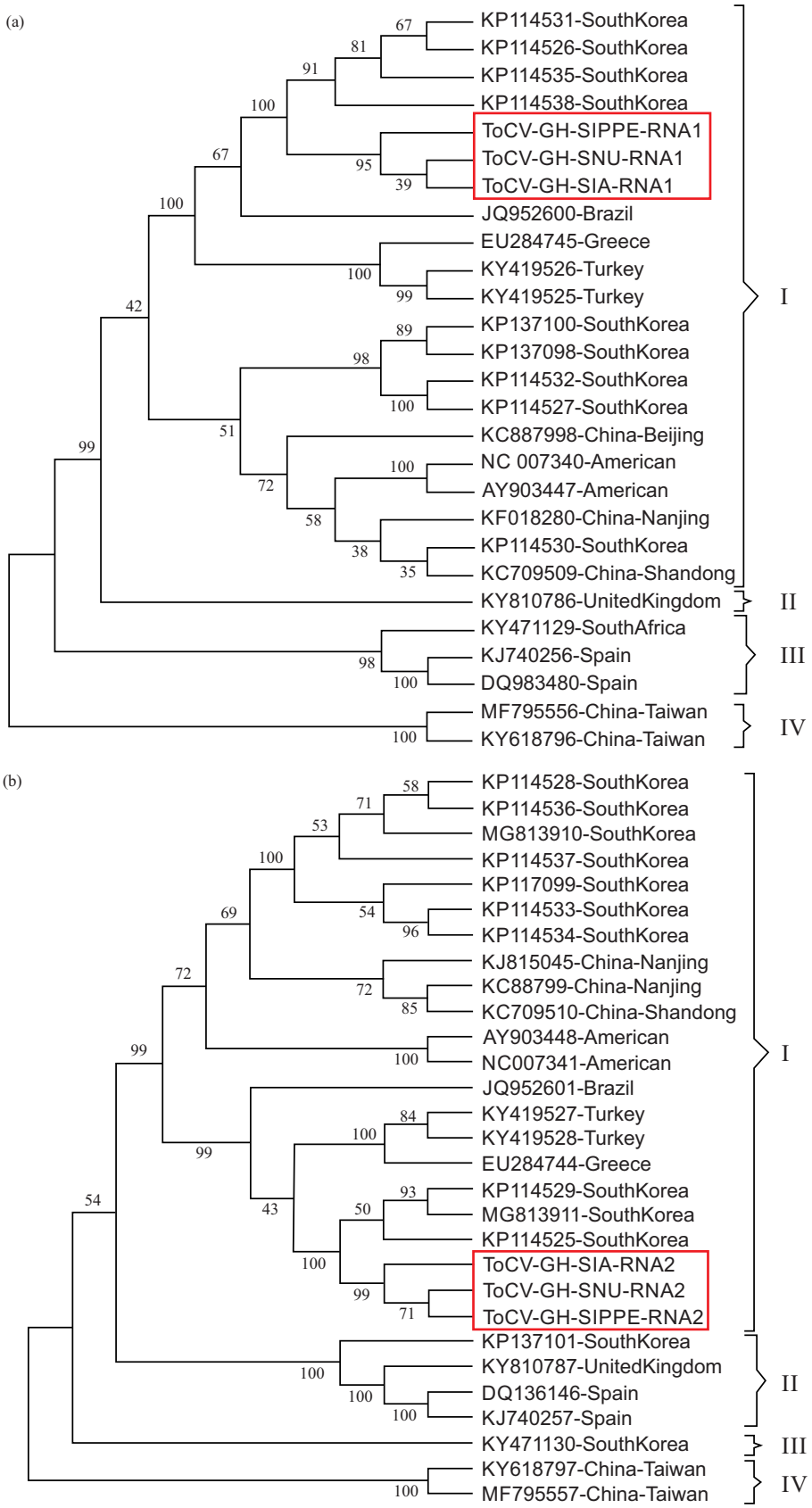

Fig. 3(a-b): Clustring analysis of (a) ToCV RNA1 and (b) ToCV RNA2

Table 2: Survey for ToCV infection in tomatoes in Shanghai

Sampling time	Sampling location	Number of samples	Number of infections	Infection rate (%)
2019/08	GH-SNU	72	50	67.2
2019/11	GH-SIA	48	42	92.1
2019/11	GH-SIPPE	45	43	94.9
2019/11	GH-SNU	27	17	62.9
2020/06	GH-SNU	42	3	7.1

RNA2 sequences in Fig. 3b showed very similar topologies, each having 4 major clusters. For both RNA1 and RNA2, the Chinese isolates, including those identified in this study, are grouped in cluster I, which consists of 2 major sub-clusters. The results showed that both RNA1 and RNA2 of the Shanghai ToCV isolates grouped closely to those from South Korea and they belong to sub-clusters that include isolates from Turkey, Brazil and Greece. Other Chinese isolates from Nanjing, Beijing and Shandong fell within another sub-cluster which included isolates from Korea and North America. The RNA1 and RNA2 of Taiwan isolate each formed a separate group in Fig. 3a and b. It is clear that in some cases, such as the isolates from the UK, South Africa and Spain, the clustering of RNA1 and RNA2 are not correlated with each other in terms of location, indicating the possibility of assortment among some isolates.

ToCV-GH-SNU-RNA1/RNA2, ToCV-GH-SIPPE-RNA1/RNA2 and ToCV-SIA-RNA1/RNA2 (red boxes) are full-length sequences obtained from the present research and the rest are from the public database (NCBI). The maximum likelihood method was used to construct the trees with the default settings in MEGA7. Both RNA1 and RNA2 can be grouped into 4 clusters (I, II, III, IV).

DISCUSSION

The present study revealed alarming rates of ToCV infection in tomato-growing greenhouses in 3 different areas of Shanghai. In the late fall, most of the tomato plants were infected with ToCV after fruits were harvested particularly in the 2 greenhouses where the greenhouse management was neglected. At this stage, most of the infected plants exhibited disease symptoms. When meticulous management was implemented in GH-SNU in the same season, the infection rate was relatively lower indicating the possible benefit of management but the infection rate of harvested samples was still above 60%. The reason for the high infection rate in this season may be that the high temperature and relatively low humidity are beneficial for plant growth but also good for whitefly reproduction. It was also one of the time points that harvested the samples for the present study and current results did show high infection rates in all the investigated greenhouses (Table 2). Nevertheless, in this study, the infection rate was much lower in the early summer during the fruit-bearing stage along with regular management. It is likely correlated with a low whitefly population in the late spring or early summer after a long winter, which makes it easier for pest control. So far, no tomato cultivar has been identified to be ToCV resistant, thus seasonal planting combined with strengthened pest control remains to be the only effective method for disease control.

It is known that the lower old leaves normally appear yellowish and chlorotic 3 weeks after the plants were infected by ToCV[16]. It was found that the association of yellowish leaves with ToCV infection was high when fruits were harvested in the late summer or late fall. However, current results indicate that it is difficult to identify infected plants at the early infection stage simply by observation. In the late spring when plants were flowering and bearing fruit, many asymptomatic plants tested positive for ToCV. In addition, yellow leaves are often ignored because they can be caused by abiotic stress, while, tomatoes infected with other viruses sometimes show symptoms of yellowish leaves[17]. Under these circumstances, different measures will be taken for growth management or disease control other than ToCV infection. Thus, to prevent large-scale ToCV infection, it is necessary to monitor the whitefly population and run a routine molecular diagnosis.

Interestingly, ToCV isolates identified by this study are closer to isolates from South Korea than to ones previously reported in China[11,18]. Thus, ToCV isolates from South Korea and Shanghai may share the same origins. The apparent spread of ToCV between Shanghai and South Korea may reflect their geographical proximity and increased agricultural trade between the 2 countries. The ability of ToCV to infect as many as 85 different plant species including some very common economic crops further increases the chance of direct spread between Shanghai and South Korea[19]. So far, ToCV has been found on every continent except Australia and New Zealand. The entry and exit management of biological products in these regions are very strict and they benefit from geographic isolation, thus such control at borders is an important factor for curbing plant diseases.

In China, ToCV appears to be widespread. The ToCV infection was reported in the greenhouses of Jiangsu Province in 2010, a neighbouring province to Shanghai[13]. It is therefore, not surprising to find it becoming a serious problem in the growth facilities in Shanghai. Although relatively few provinces have been reported, given the wide host ranges of ToCV and whitefly transmission, the problem is likely much more widespread than realized. It is therefore, important to monitor the viral distribution and to initiate strategies for virus prevention.

CONCLUSION

The infection rates are about 63-95%. The full-length sequences of the 3 isolates were obtained and exhibit high sequence identity. They all share 99.7% similarity to South Korean strains (KP114531, KP114526, KP114535, KP114538, KP114529, MG813911 and KP114525). For one of the greenhouses, the ToCV infection rates were analyzed in 3 consecutive tomato growing seasons and the lowest infection rate came from the samples harvested in late spring. The results also indicate the role of greenhouse management in disease control.

SIGNIFICANCE STATEMENT

This research used the ToCV epidemic in Shanghai growth facilities as the study case to show its seasonal changing feature and the important but limited role of greenhouse management. In the meantime, the sequence analysis suggests the Shanghai isolates may have different origins than the reported strains in China. These results will give important guidance for ToCV disease prevention and control and benefit tomato and other vegetable farming management and business.

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