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Detection and Identification of Tomato Spotted Wilt Virus in Ornamental Plants in North Khorasan Province

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Abstract

In recent years, the symptoms of viral diseases such as dwarfism, mosaic, discoloration, necrosis, and circular spots have been prevalent in ornamental plants in parks, gardens, and streets in North Khorasan Province. Tomato spotted wilt virus (TSWV), the type species of the genus *Orthotospovirus*, the family Tospoviridae, and the order Bunyavirales, is a tripartite negative/ambisense ssRNA virus. This virus is a major cause of the infection of ornamental plants in the world. In this study, to evaluate the percentage of infection to TSWV, in the early autumn of 2020, 350 samples of ornamental plants were collected based on the suspicious viral symptoms from parks, gardens, and streets of North Khorasan province (Bojnord and Shirvan cities) and were transported to the laboratory in cold conditions. In the laboratory, DAS-ELISA serological test was performed to evaluate the presence of virus in the suspected samples. Then, the infected samples identified by ELISA test were inoculated to the test plants of *Chenopodium album* L. (lamb's quarters), *Vigna unguiculata* L. (cowpea) and *Datura stramonium* L. (jimsonweed). After the appearance of symptoms, in order to verify the infection, they were tested again using DAS-ELISA. The molecular identification of the infected samples was done by Qiagene RNA extraction kit. Using specific primers in RT-PCR reaction, a fragment was amplified in the band of 276 bp. The results of DAS-ELISA and RT-PCR tests proved the presence of the virus on ornamental plants in North Khorasan province. In the mechanical inoculation of the virus, three above-mentioned plants showed the symptoms of the disease.

Keywords: DAS-ELISA, Mechanical inoculation, Ornamental plants, RT-PCR, Viral diseases.

INTRODUCTION

The area under the cultivation of ornamental plants in the world is approximately 560,000 ha and the area of greenhouses for their cultivation is 120,000 ha (Anonymous, 2021). Iran is a country that has four seasons with climate variability. Therefore, there are good potentials for the production of flowers and ornamental plants in Iran (Mostashar Nezami *et al*., 2013). A large part of Iranian territory in the north, center, and south-west of the country is suitable for developing a variety of ornamental plants and flowers (OPRC, 2023). The total area under the cultivation of ornamental plants in Iran including greenhouses and outdoor areas is 7,798 ha, which is of economic importance (Anonymous, 2018). In recent years, flowers and ornamental plants industry in Iran has developed extensively (Mahmoodi Safa *et al*., 2016). Ornamental plants are grown in all provinces of Iran and can be classified into four groups of cut flowers, potted flowers, trees and shrubs, and seasonal and transplanted plants. The ornamental plants are cultivated performed in both outdoor areas and greenhouses (Mahmoodi Safa *et al*., 2015).

Viruses are the most common pathogenic factors in many ornamental flowers, but they are often overlooked because their symptoms are mild or there are no symptoms at all. The viral diseases are often asymptomatic and do not pose a problem, but the problem occurs when the virus had been transmitted to a susceptible host and has caused severe damages and even death. In fact, this asymptomatic factor acts as a source of the pathogen (Mathews, 2010). Plant diseases caused by infection with tospoviruses have been causing enormous crop losses worldwide (Oliver and Whitfield, 2016). The *Tomato Spotted Wilt Virus* (TSWV), the type species of the genus *Orthotospovirus*, the family Tospoviridae, and the order Bunyavirales, is a tripartite negative/ambisense ssRNA virus (Zhang *et al*., 2021). Based on their molecular weight, the three ssRNAs have been designated RNA-S, RNA-L, and RNA-M. RNA-L is the negative sense RNA, which encodes RNA-dependent RNA polymerase (RdRp). RNA-M and RNA-S are ambisense RNAs, which encode the movement protein NSm and the glycoprotein Gn/Gc, respectively. The TSWV has a worldwide distribution and infects more than 1,000 species of plants in more than 80 families (Pappu *et al*., 2009).

The TSWV is transmitted in a persistent propagative manner by western flower thrips. Thrips acquire the virus during larval stages, and only thrips adults that acquired the virus as larvae can transmit the pathogen (Oliver and Whitfield, 2016). The characteristic symptom of the TSWV is the appearance of obvious concentric rings on the leaves and fruits, which later turn brown. The leaves may appear chlorotic and bronze, and premature senescence starts from the old leaves (Wangai *et al*., 2001).

Tomato Spotted Wilt Virus is widely spread in Iran. This virus has been reported from different regions of Iran from tomato and soybean hosts in fields and greenhouses (Golnaraghi *et al*., 2008; Massumi *et al*., 2009; Farzadfar *et al*., 2002). It has been reported from different regions of Iran on the variety of ornamental plants like *Anemone petiolulosa* L., *Vinca minor* L., *Cineraria cruentus* L. and *Tropaeolum majus* L. (Ayazpour, 2014). Therefore, it is necessary to know the contamination level of the ornamental plants to the virus in Iran and to determine its prevalence in different areas in order to properly manage and control it on a large scale.

To prevent the losses caused by plant viruses, it is necessary to develop specific and efficient diagnostic tools to detect viruses. Among the current virus detection techniques, serological and molecular detection methods are considered to be rapid, simple, sensitive, and high throughput. Therefore, serological and molecular detection methods such as double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and reverse transcription polymerase

chain reaction (RT-PCR) are now wildly used to detect viruses in plants. Given the history of tomato spotted wilt virus in various agricultural products and ornamental plants in Iran, easy transmission, wide host range of the virus, and viral-like symptoms on leaves of ornamental plants in some areas located in province, attempts were made in this research to identify and detect the virus contamination in various ornamental plants in some cities of North Khorasan Province and finally, the percentage of TSWV infection was investigated.

MATERIALS AND METHODS

Sampling

In the early autumn of 2020, 350 samples of ornamental plants (175 samples from each city) were collected from parks, gardens, and streets in Bojnord and Shirvan cities in North Khorasan Province. The samples included different plant species based on the suspicious viral symptoms collected at the time of sampling. They were from 9 families and 16 species of *Viola* sp. L., *Matthiola incana* L., *Gaillardia pulchella* Foug., *Calendula officinalis* L., *Chrysanthemum indicum* L., *Zinnia elegans* L., *Leucanthemum* × *superbum*., *Ipomoea tricolor* Cav., *Ipomoea batatas* (L.) Lam., *Rosa hybrida* L., *Pelargonium* × *hortorum*., *Amaranthus caudatus* L., *Lepidium draba* L., *Berberis Thunbergii* L., *Bellis perennis* L., and *Galium odarata* L. They included leaves and young stems of ornamental plants with different types of viral-like symptoms such as wanness, necrosis, chlorosis, mosaic, and ring stains or without obvious symptoms and were transported to the laboratory in cool conditions.

Detection and identification of TSWV

Double antibody sandwich ELISA (DAS-ELISA) was used for TSWV detection according to Clark and Adams (1977) method. In the test, the commercial kits of polyclonal antibodies (Bioreba, Switzerland), (dilution of 1000) were used to detect the virus. ELISA tests for the detection of the virus were done according to the manufacturer's instructions as follows: Immunoglobulin (Virus-specific IgG) with coating buffer to the ratio of 1: 1000 was mixed and 100 ml was poured into each well. Then, the collected leaf samples were extracted at the ratio of 1:5 (1 g of leaf tissue in 5 ml of the extraction buffer) in the cold porcelain mortar. After adding the extract to the ELISA plate, IgG -AP was again diluted with the same ratio (1: 1000) by the conjugate buffer and added to the wells.To change the color of the wells, para-nitro-phenyl phosphate substrate (PNPP) was used. The plates were washed three times with PBS-Tween buffer. In this test, the color-forming reaction of the positive samples and the presence of yellow color were evaluated using ELISA reader (ELX 800-Biotek model) at 405 nm. The calculations were done and the positive samples were determined using the formula R = x + 3 SD, where x= attraction mean of negative samples, SD = standard deviation of wells, and R = threshold level of pollution.

Inoculation to test plants

In order to see the symptoms and pathogenicity of the virus, the infected samples that were identified in ELISA were inoculated to the test plants. Profile and age of the inoculated plants are given in table 1. The plants were planted directly. First, the test plant seeds were planted in medium-sized plastic pots composed of two layers of sand, two layers of peat, and one layer of manure. Then, they were kept in the greenhouse under the appropriate conditions of temperature, humidity, and light. To inoculate the test plants, the inoculation buffer containing

potassium phosphate buffer (including K_2HPO_4 and KH_2PO_4), (0.01M with pH=7), which contained 2-mercaptoethanol (0.15 %), was prepared. The infected samples placed in the Chinese mortars were extracted with 2 ml of cold incubation buffer; then, carborundum was sprayed on the leaves to enhance the penetration of the virus and extract. Then, separately using the index finger, the leaves were inoculated (parallel to the veins, especially the central vein.) and, 3 min after inoculation, the leaves were washed to remove extra carborundum. Inoculation was done during the cool hours of the day (in the morning or at evening). The inoculated plants were kept at 25-30 °C and indirect lighting in the greenhouse and, in order to prevent the insects carrying the virus, lacing was used around the pots. These pots were irrigated regularly and were visited to record the progress of work each day. After the symptoms appeared, all the plants were tested by DAS-ELISA to confirm the contamination.

Table 1. Test plants inoculated with virus and their inoculation age.

Test plants	Scientific name	Inoculation age
Lamb's quarters	*Chenopodium album* L.	2 to 4 leaves
Cowpea	*Vigna unguiculata* L.	2 to 4 leaves
Jimsonweed	*Datura stramonium* L.	2 to 4 leaves

Molecular detection of TSWV

For verification and closer examinations, the infected samples were evaluated in ELISA by RT-PCR test.

RNA extraction and cDNA synthesis

Since the genome of the virus is of RNA kind, by reverse transcription, RNA was changed to DNA and, then, PCR amplification was done on the obtained cDNA. RNA extraction was performed using Qiagen kit (Qiagen, United Kingdom). The absorbed RNA was used for reverse transcription reaction using AccupowerTM RT Premix kit (Bioneer, South Korea). The process was as follows:

A. First, 6 µl of the extracted RNA and 1 µl of the reverse transcriptase enzyme were poured into 0.5 ml microtube.

B. Then, the micro-tubes were placed inside the heater block for 5 min at 70 °C.

C. All the contents of the micro-tubes were transferred by the pipette into 0.5 ml microtubes of the kit. Then, 13 ml of the distilled water was added to have 20 ml of reaction volume.

D. Finally, the micro-tubes were centrifuged to be a little monotonous and, then, were placed in the thermocycler at 42°C for 1h for cDNA synthesis and 94 °C for 5 min to deactivate RNase.

E. The micro-tubes were removed from the device and stored in the freezer at 20°C.

Polymerase chain reaction (PCR)

For this purpose, two pairs of specific primers derived from TSWV genome RNA-L were used (Table 2).

These primers reached the appropriate concentration by autoclaved double sterilized water and stored in the freezer at -20°C. After the RT reaction and cDNA synthesis, the PCR reaction was done for cDNA amplification using AccupowerTM RT Premix kit in accordance with the following instructions:

A. PCR micro-tubes contained some of the materials needed, such as PCR buffer, MgCL2, dNTPS, and Taq polymerase enzyme for polymerase chain reaction. These micro-tubes were selected and named for testing; then, 5 µl of the cDNA was poured into the kits.

B. 1 ml of each of the primers was added to the kit.

C. At last, 13 ml of the distilled water was added to the micro-tubes and to make the final reaction volume of 25 µl (Table 3).

Table 2. Profile of the primers used in polymerase chain reaction to amplify the viral genomic RNA-L.

Virus	Name of primers	Directions	Sequence of primers	References
TSWV	L 2 TSWV-F	Forward	ATCAGTCGAAATGGTCGGCA	Morris (2004)
TSWV	L 1 TSWV-R	Reverse	AATTGCCTTGCAACCAATTC	Morris (2004)

Table 3. Ingredients and their dosage to prepare a PCR solution.

Materials	The dosage used in each 25 µL reaction volume (µl)
Buffer PCR (10X)	2.5
$MgCl_2$ (50 Mm)	1.5
dNTPS (10 Mm)	0.5
cDNA	5
Taq DNA polymerase (5 U/µ)	0.3
Forward-primer (10 µM)	1
Reverse-primer (10 µM)	1
Deionized water	13.2

Table 4. Step of PCR test for TSWV detection.

Thermal cycle	Number of cycles	Temperature (°C)	Duration (min)
The initial denaturation	1	94	5
Denaturing		94	1
Gradual connection	38	50	1
Extension		72	1
A final extension	1	72	10

D. The micro-tubes were centrifuged at low speed until the ingredients were well mixed and did not stick to the walls of the micro-tubes.

E. The micro-tubes were placed in the thermocycler device, the program of which is already given (Table 4).

To maintain PCR product, the end cycle of the reaction after the final expansion stage was set at 4 °C for 1h in all the programs.

Agarose gel electrophoresis

Agarose gel electrophoresis 1% in TBE 1X buffer was used to check the results of PCR. For buffer TBE 10X, 10.8 g Tris, 5.5 g boric acid, and 0.37 g EDTA were dissolved in 100 ml of distilled water and its acidity and pH were adjusted to 8.3 and (1x) diluted when being used. To prepare the gel, 0.5g agarose was dissolved in 50 ml of TBE 1X buffer. Agarose must be completely dissolved in the buffer to have a clear solution. After the solution was cooled slightly at room temperature, 5 ml of pre-prepared SYBR Green solution (EURX, Poland) prepared was added and stirred well to completely solve SYBR Green in the agarose solution. Then, the solution was poured into the tray for molding the gel and it was placed inside the

electrophoresis so that the wells were placed next to the negative electrode. After 20 min, the comb was removed from the gel and the reservoir tank was filled with TBE 1X buffer so that the gel was completely covered and the wells were ready to be poured into the samples. Then, 5 ml of the extracted RNA was mixed with 2 ml of the dye solution and poured into the wells. The electrophoresis voltage was set to 90V and 60 min and the size was determined using the standard marker of 100 bp ladder to ensure the expected size and accuracy of the amplification reaction. After decolorization with distilled water, the gel was examined on a UV Transilluminator and, then, was photographed using gel documentation.

RESULTS

ELISA serological test results

Results of 350 samples of the collected ornamental plants showed high prevalence of virallike symptoms in the plants in North Khorasan province (Fig. 1). In this study, 350 samples of 16 species of ornamental plants were tested for *Tomato Spot Wilt Virus* (TSWV), which is an important and economic viral disease with global prevalence. Most of the tested plants were ornamental or used in outdoor gardens.

Results of ELISA serological test showed that among 350 ornamental tested plant samples, 37 samples were infected with the virus (10.57%) and 313 samples (89.43%) had no virus infection (Fig. 2). Results also demonstrated that the virus existed in both cities of Bojnord and Shirvan. TSWV infections in the city of Shirvan were 8% and it was 2.57% in Bojnord city. Out of 16 species of the ornamental plants, 3 species were infected and 14 species were without infection to TSWV. TSWV was identified in 14 samples of *Chrysantheum indicum* (L.) Lam, 7 samples of *Zinnia elegans* (L.) Lam. and 16 samples of *Rosa hybrida* L.

In the mechanical inoculation of TSWV, the entire mentioned test plants showed the symptoms of the disease (Fig. 3). In addition, it was observed that, in most cases, the symptoms of the plants were worse in warmer conditions. ELISA confirmed the contamination of all the test plants inoculated with the virus.

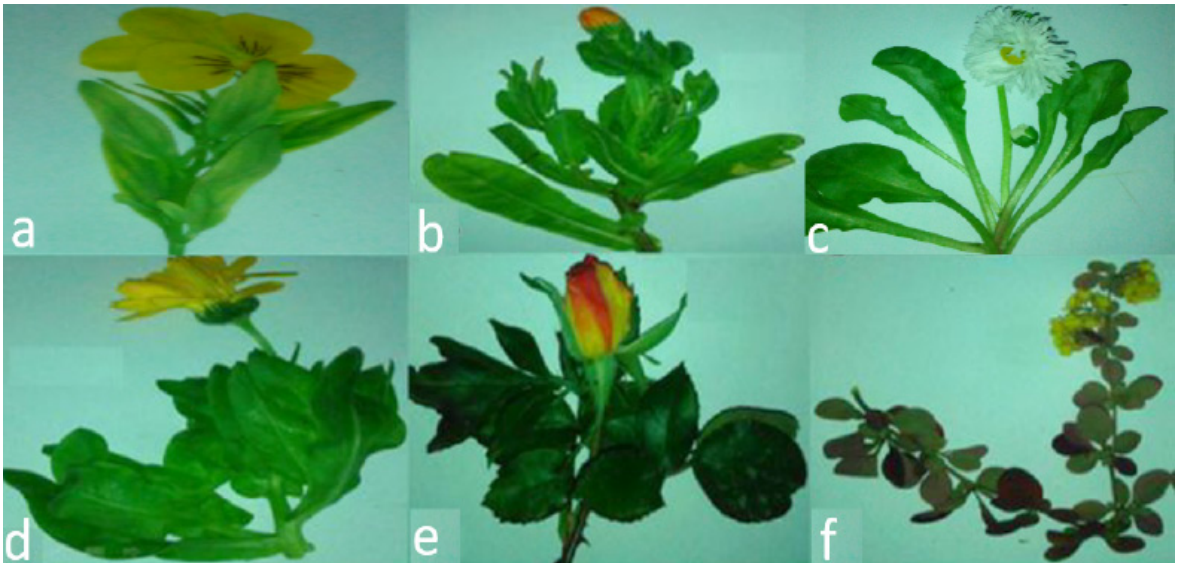

Fig. 1. Viral-like symptoms in ornamental plants in North Khorasan Province. (a) Uniform chlorosis symptoms on *Viola* sp. L. (b) Necrotic spots symptoms on *Calendula officinalis* L. (c) Malformation symptoms on *Chrysantheum indicum* L. (d) Severe mosaic symptoms on *Calendula officinalis* L. (e) Pale spots symptoms on *Rosa hybrida* L. (f) Discoloration symptoms on *Berberis thunbergii* L.

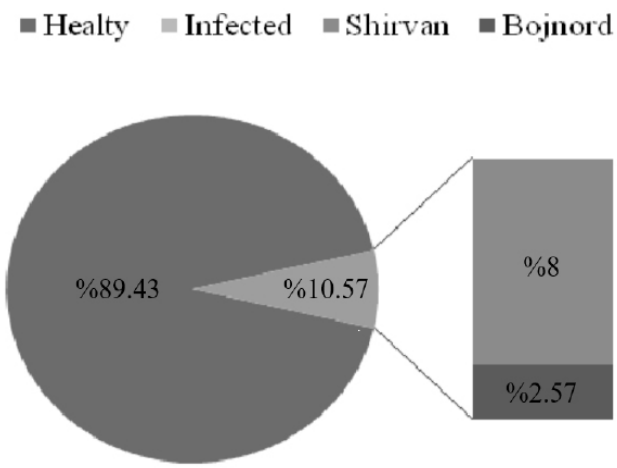

Fig. 2. Infection percentage of ornamental plants to TSWV in North Khorasan province.

RT-PCR test results

The results of RT-PCR using specific primers to detect TSWV are presented in Fig. 4. In this way, a region of the TSWV genome RNA-L was amplified and a fragment of 276 bp of the infected *Chrysantheum indicum* (L.) Lam. sample was obtained. These tests conclusively proved the presence of TSWV in the province.

Fig. 3. Symptoms of infection to TSWV. (a) Systemic chlorosis in *Datura stramonium* L. (b) Systemic yellow vein of leaves in *Vigna unguiculata* L. (c) Mild to severe chlorosis in *Datura stramonium* L. (d) Local necrotic spots and Systemic mosaic in *Chenopodium album* L.

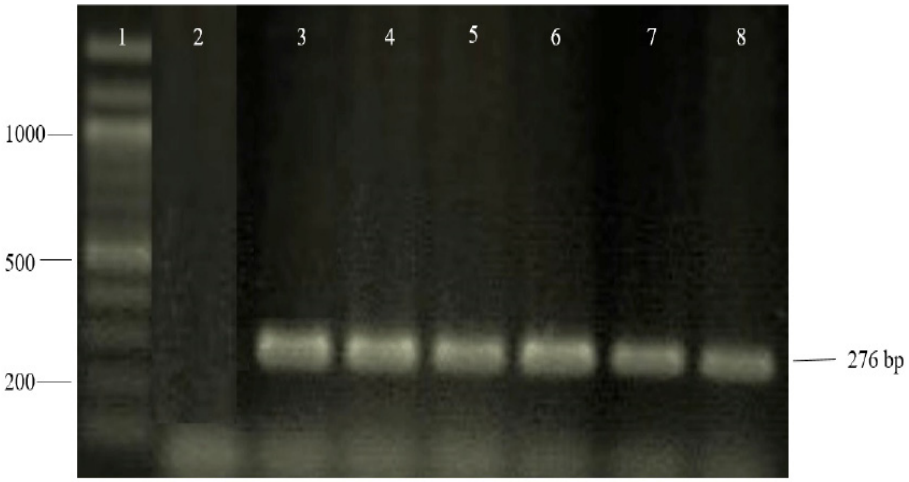

Fig. 4. Electerophoresis pattern of RT-PCR products of tomato spot wilt virus. Lane 1, Gene ruler DNA ladder 100 bp; Lane 2, Healthy ornamental plant; Lane 3-8, *Chrysantheum indicum* (L.) Lam. Samples infected.

DISCUSSION

Tomato Spotted Wilt Virus (TSWV), that can infect tomatoes and a variety of plants in Iran, is among the most damaging and important viruses. Most of the reports of contamination of ornamental plants in the north of the country are related to TSWV, INSV and CMV viruses, and TSWV has the highest percentage of contamination in ornamental plants in the northern provinces of Iran (Ghotbi and Shahraeen, 2012). In this study, the prevalence of TSWV in parks, gardens, and streets of the cities in North Khorasan province was investigated (Bojnord and Shirvan). The results of this study are the first evidence of the presence of TSWV in ornamental plants of North Khorasan province. The prevalence of TSWV in ornamental plants can be justified given the suitability of environmental conditions for the activity of western flower thrips, inefficient use of certified virus-free seedlings, and lack of laboratory facilities in the quarantine posts and contamination reports of tomato fields in North Khorasan Province to TSWV.

In this study, the presence of the virus in the ornamental plant of *Chrysantheum indicum* (L.) Lam. was confirmed using classical and molecular biological methods. Although, various researchers in Iran have performed the molecular detection of virus on different crops, this work is the first report of molecular detection of TSWV in ornamental plants in Iran. RT-PCR test is more sensitive than ELISA. Also, RT-PCR is simpler than other molecular methods and, thus, a large number of samples can be tested, simultaneously. The quality of total nucleic acid and extraction method is very important as well (Gumus and Paylan, 2013). In this study, there were infected samples with positive ELISA test results, but in most cases, in RT-PCR test, there is no proliferation using specific primers of virus. The non-positive results of RT-PCR using ToRSV-specific primers can have a variety of reasons such as low quality of nucleic acid, inappropriate RNA extraction method, and even false coloring of the wells in ELISA test for various reasons. It seems that the use of more advanced molecular methods, especially IC-PCR method in future research, can reduce the abovementioned problems due to the use of both serological and molecular methods in one-step. In addition, due to the high concentration of the virus, IC-PCR method can be useful in the detection of the virus with the same symptoms on the test plants.

Although, ELISA is better than RT-PCR test due to such advantages as no need for

special education and economic factors, RT-PCR method is important and can contribute to viral disease management programs owing to the unique characteristics such as simplicity, fastness, and traceability of virus even at low concentrations in genetic engineering for determining viral pathogens. In the mechanical inoculation of TSWV on the test plant of *Nicotiana tabacum* var. *Samson*, Shoshtari *et al*. (2013) observed the Mosaic in leaves, which corresponded to the results of the *Chenopodium album* test plant in response to TSWV in this study. Also, a large number of symptomatic plants did not react to the antibodies of TSWV. The lack of positive reaction might be due to contamination with other common viruses such as TMV, TuMV, BYMV, or latent viruses (Ghotbi and Shahraeen, 2012). Damages caused by viral infections in plants, can vary from 10 to 100% depending on the composition of the virus and host (Loebenstein *et al*., 1995). The most important approach against viral diseases is to prevent their spread. Since the propagation method of most plants is vegetative, in this regard, secure and virus-free seedlings, bulbs, and vegetative parts are the effective method for preventing the spread of viral diseases in ornamental plants. Therefore, as an ongoing need, there is a need to launch and develop a virus-free seedling production system in the country by producers.

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