Effects of Temperature and Host on the Generation of Tomato Spotted Wilt Virus Defective Interfering RNAs

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ABSTRACT

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The generation of defective interfering (DI) RNA molecules of tomato spotted wilt tospovirus (TSWV) was studied by serially passaging inocula from plant to plant under different controlled conditions. DI RNAs were generated at higher rates in plants at 16°C than in plants incubated at higher temperatures. Another factor promoting the TSWV DI RNA generation was the use of high virus concentrations in the inocula. The solanaceous species *Capsicum annuum*, *Datura stramonium*, *Lycopersi-*

con esculentum, *Nicotiana benthamiana*, and *N. rustica* supported the generation of DI RNAs, whereas the virus recovered from the inoculated composite species, *Emilia sonchifolia*, remained free of any DI RNA under all conditions tested. This study resulted in a strategy to maintain DI RNA-free TSWV isolates, as well as in an efficient way to produce a large population of different DI RNA species. A single DI RNA species usually became dominant in an isolate after a few rounds of serial inoculations. The possible mechanisms involved in TSWV DI RNA generation under different inoculation circumstances are discussed.

Additional keywords: *Bunyaviridae*, host plant species, virus concentration in inoculum.

Defective interfering (DI) RNA molecules, reported for a considerable number of viruses (2,20,25), arise spontaneously by deletions and rearrangements of the viral genome during infection. They are packaged like wild-type RNAs and interfere with the replication of the wild-type virus, i.e., they replicate dependently and preferentially at the expense of the nondefective parental virus (12).

The existence of DI particles was first reported in influenza virus (29) and, subsequently, extensively described in several animal viruses (2,13,20). In plant viruses, the first DI RNA was found in tomato bushy stunt tomosvirus "cherry" strain (TBSV-Ch, also known as petunia asteroid virus [11]). After this report, peculiarities on the conditions of de novo generation of DI RNAs of several viruses were reported, e.g., in the *Bromovirus* (21), *Carmovirus* (18), *Furovirus* (27), and *Tombusvirus* genera (3,4,14,24).

It is generally accepted that DI RNAs originate as a consequence of serially passaging a virus at high multiplicity of infections through a susceptible host (25,26). A high inoculum concentration was demonstrated to be essential for the generation of DI RNAs of cucumber necrosis tomosvirus (CNV [24]), cymbidium ringspot tomosvirus (CymRSV [4]), and TBSV-Ch (14). At least eight passages were required before the detection of DI RNAs in CNV-inoculated plants (24), whereas DI RNAs could already be detected after the third passage in CymRSV- (4) and TBSV-Ch-infected (14) plants. When TBSV-Ch was inoculated in low concentrations, DI RNAs could not be detected after 11 serial passages, suggesting a strong effect of the virus concentration in the inocula on the generation of DI RNAs (14). Factors such as the incubation temperature applied to the inoculated plants, as shown for soilborne wheat mosaic furovirus (6), or the host plant used, when inoculated with CNV (5), were also reported to modulate the rate at which the DI RNAs were generated and accumulated in

the infected plant. It is believed that a complex of factors contribute, either in- or interdependently, to the process of DI RNA formation and accumulation.

DI RNAs of tomato spotted wilt tospovirus (TSWV) were recently detected in serial mechanically inoculated plants using highly concentrated inocula (22). TSWV, the type species of the genus *Tospovirus* of the family *Bunyaviridae*, is an enveloped virus containing one negative-sense RNA, large (L) RNA, and two ambisense RNA segments, medium (M) and short (S) RNA, with respective sizes of 8,897, 4,821, and 2,916 bases (8,9,15). The DI RNAs of TSWV, ranging in size from 3.1 to 3.4 kb, were all derived from the L RNA (22,23), which encoded the putative RNA dependent RNA polymerase (8,28). These DI RNAs were consistently associated with disease symptom attenuation in infected plants (22).

In this study, aimed to establish strategies to maintain DI-free TSWV isolates after mechanical inoculation of plants and to produce a large number of distinct DI RNAs, the effect of different inoculation and growing conditions on the generation of DI RNAs was investigated. The analysis demonstrated a striking influence of the temperature, host species, and virus concentration in the inocula on the generation and accumulation of DI RNAs.

MATERIALS AND METHODS

Virus, plant species, and inoculation procedures. A DI-free stock of the Brazilian wild-type TSWV isolate BR-01 (7) was used in this study to inoculate a first set of three plants. Each plant in this set was used to start a series of plant lines that were successively inoculated with an extract prepared from the last inoculated plant in a line. The first inoculum was prepared by homogenizing systemically infected *Nicotiana rustica* L. leaves in 0.01 M phosphate buffer, pH 7.0, containing 0.01 M sodium sulfite. After inoculation, the plants were incubated in climate chambers with a daily light regime of 16 h at a constant temperature of 23°C, except when the effect of temperature was tested.

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Serial inoculations were made with extracts from systemically infected leaves harvested at a predetermined postinoculation incubation time (Table 1). This period corresponded with the minimum required time for the accumulation of maximal amounts of viral antigen in the infected plants. The virus concentration at this stage was determined by enzyme-linked immunosorbent assay (ELISA), and leaf extract dilutions were adjusted to provide inocula with equal virus concentrations for serial inoculations (Table 1). When a delay of the symptom development occurred during serial passages, the harvesting date was postponed to the second day after the appearance of systemic symptoms. This date usually corresponded with the highest concentration of viral antigens in the plant. Each series consisted of 15 passages.

From every plant, systemically infected leaves were harvested, immediately used as inoculum for the subsequent inoculation, or divided in aliquots and stored at -70°C until further analysis.

Plants to be inoculated were always used in the same developmental stage. *Datura stramonium* L. and tomato (*Lycopersicon esculentum* Mill.) cv. Moneymaker were used in the second-leaf stage; *N. rustica* cv. America and *Emilia sonchifolia* (L.) DC. ex Wight in the third-leaf stage; sweet pepper (*Capsicum annuum* L.) cv. Lambada in the fourth-leaf stage; and *N. benthamiana* Domin. in the fifth-leaf stage.

ELISA. The viral antigen concentration in the systematically infected leaves was estimated by double-antibody sandwich-ELISA using polyclonal antibodies against the nucleocapsid (N) protein. Leaf disks of 5 mm in diameter were collected from the plants using a one-hole puncher and stored frozen at -20°C . Before plating, each disk was crushed in 0.01 M potassium phosphate buffer, pH 7.2, containing 0.14 M NaCl and 0.05% Tween-20 (PBS-T) in a dilution of 1 mg of leaf/ml of PBS-T. This high dilution was used to avoid saturation of the antigen-antibody reaction. Each sample was tested in duplicate using 100 μl of extract per well.

Northern blotting. To analyze the presence of L RNA-specific RNA species, total RNA extracts of systemically infected leaves were tested by Northern blot hybridization. The analysis was performed after the fifth, tenth, and fifteenth passage. These extracts were prepared using the sodium dodecyl sulfate-sodium acetate method (10), except for *N. benthamiana*, for which a method with guanidium hydrochloride (19) was used. The RNA from 0.1 g of systemically infected leaf material was finally resuspended in 25 μl of H_2O . A 5-ml aliquot of each extract was denatured with methylmercuric hydroxide (1), subjected to electrophoresis in a 1% agarose gel using a borate buffer (50 mM boric acid, 5 mM sodium borate, and 10 mM sodium sulfate without addition of methylmercuric hydroxide), pH 8.1, and transferred onto a nylon membrane (Gene-screen NEF 972; DuPont NEN, Dordrecht, the Netherlands). Northern blot hybridization was performed with a mixture of two distinct double-stranded-DNA probes that were ^{32}P -labeled by random priming. One probe was specific to the 5' termini (nucleotide positions 1 to 1,178) and the other to the 3' termini (nucleotide positions 7,749 to 8,897) of the reported viral complementary (vc) strand of TSWV L RNA (8).

Reverse transcription and polymerase chain reaction (PCR). First-strand L RNA-specific cDNA was synthesized from total RNA extracts using the primer pDH001 (CCCGGATCCTCGAG-AGCAATCAGGTAACA, identical to the first 17 nucleotides of the vc strand of TSWV L RNA [8]). Prior to PCR amplification and subsequent cloning, cDNA was treated with 1 unit of RNase H (Gibco BRL, Gaithersburg, MD) at 37°C for 20 min. PCR amplification was performed according to manufacturer procedures using *Taq* Extender PCR Additive (Stratagene Inc., La Jolla, CA) using pDH001 and pDH002 (CCCGTCGACTAATACGACTCA-CTATAGAGCAATCAGGTACAA, complementary to nucleotides 8,880 to 8,897 of the vc strand of TSWV L RNA [8]). Each of the 40 cycles of PCR amplification consisted of 30 s of denaturation at 94°C , 1 min of annealing at 52°C , and 4 min of extension at 72°C .

PCR products were subjected to electrophoresis in 1% agarose gel containing ethidium bromide.

RESULTS

Determination of incubation time and dilution rate for serial inoculations. To study the factors involved in the generation of DI RNAs, total control of inoculation conditions was required. To provide more accuracy during serial inoculations, the infection development was studied by monitoring the incubation time and establishing the dilution of leaf extracts to be used (Table 1). Leaf disks were collected daily after inoculation and tested by ELISA using antibodies against N protein. Accumulation of virus was invariably sigmoidal with time. After a lag period, the virus accumulated at a fast rate and reached a plateau that remained stable throughout the evaluation time of 32 days (data not shown).

The minimum time required for accumulation of the maximal amount of viral protein varied with the conditions applied (Table 1). The ELISA values recorded after the incubation period are shown in Table 1 as the mean value of five replicates. The small variation of the ELISA values found in the five solanaceous hosts indicated the presence of similar virus concentrations in the leaves under all test conditions. The only exception was the *E. sonchifolia* plants, which had an approximately two times lower virus concentration (Table 1). Based on these results, a plant extract dilution of five times was chosen to inoculate the solanaceous hosts, and two times when *E. sonchifolia* was tested. This provided inocula with approximately equal virus concentrations in the successive serial passages (Table 1).

Effect of host plant on the generation of DI RNAs. To investigate whether different systemic host plant species affect the formation and accumulation of DI RNAs of TSWV, the solanaceous species *C. annuum*, *D. stramonium*, *L. esculentum*, *N. benthamiana*,

TABLE 1. Accumulation of virus antigen in systemically infected hosts after applying different inoculation and growing conditions

Tested condition	Incubation ^a	ELISA ^b	Dilution ^c
Host ^d			
<i>Nicotiana rustica</i>	8	0.33 ± 0.05	5
Tomato	9	0.25 ± 0.04	5
Sweet pepper	9	0.30 ± 0.01	5
<i>Nicotiana benthamiana</i>	7	0.23 ± 0.02	5
<i>Datura stramonium</i>	8	0.30 ± 0.02	5
<i>Emilia sonchifolia</i>	8	0.14 ± 0.02	2
Temperature ^{e,f}			
16°C	12	0.40 ± 0.04	5
30°C	7	0.28 ± 0.01	5
Dilution ^{e,g}			
50	8	0.27 ± 0.02	...
1,000	9	0.25 ± 0.02	...
Incubation time ^{e,h}			
16 days	...	0.33 ± 0.04	5
32 days	...	0.30 ± 0.05	5

^a Incubation time (days) required for maximum accumulation of nucleocapsid protein.

^b Mean enzyme-linked immunosorbent assay (ELISA) values (405 nm) of five replicates obtained from extracts of leaves collected at the incubation time^a. Standard error deviations are indicated.

^c Dilution of leaf extract employed for serial inoculations (wt/vol).

^d Plants were inoculated with five times diluted leaf extracts and incubated at 23°C .

^e *N. rustica* plants, inoculated with five times diluted leaf extracts and incubated for 8 days at 23°C , were part of these comparative studies.

^f Incubation temperature of *N. rustica* plants inoculated with five times diluted leaf extracts.

^g Dilution of leaf extracts (wt/vol) before inoculating *N. rustica* plants and incubating at 23°C .

^h Age of the infection in *N. rustica* plants inoculated with five times diluted leaf extracts and incubated at 23°C .

N. rustica, and the composite species *E. sonchifolia* were inoculated with TSWV during serial passages.

Total RNA extracts from infected leaves collected after the fifth passage were analyzed by hybridization using L RNA-specific probes (Fig. 1A). In addition to the full-length L RNA, smaller RNA molecules could clearly be detected in one tomato line (To-2), in two sweet pepper lines (Pe-1 and -2), and in two *D. stramonium* lines (Da-1 and -2). They were not detected in any *N. rustica* (23°C-5), *N. benthamiana* (Be), and *E. sonchifolia* (E) plants.

These smaller RNAs did not represent breakdown products of the L RNA, since they were PCR-amplified using primers specific to both genomic ends of the L RNA. Total RNA extracts from plants infected with TSWV DI RNAs were reverse-transcribed and subjected to PCR amplification. Electrophoresis of these PCR products revealed DNA fragments with the expected sizes, as estimated on Northern blots (Fig. 2, DI Pe-1, Pe-2, and Da-7). These defective RNA molecules will be further referred to as DI RNAs.

In addition to the DI RNAs found in the fifth passage, after the tenth passage (Fig. 1B), DI RNAs were detected in two *N. rustica* lines (23°C-5-2 and -3), in one *N. benthamiana* line (Be-3), and in one sweet pepper line (Pe-3). Once more, DI RNA molecules were not detected in any of the *E. sonchifolia* lines analyzed.

DI RNA molecules were observed in all the solanaceous hosts analyzed after the fifteenth passage (Fig. 1C), except for one *N. rustica* (23°C-5-1), one tomato (To-1), and one *N. benthamiana* line (Be-2). No DI RNAs were detected in any of the three *E. sonchifolia* lines.

The first DI RNA was detected in a *D. stramonium* line (Da-1) during the third passage (data not shown), whereas, in most other cases, DI RNAs were found during the fifth to fifteenth passage (Fig. 1). Analysis of the migration rate of DI RNAs in Northern blot hybridization showed that DI RNAs, once detected, were

preserved in subsequent passages. In three lines, however, the DI RNAs originally found were clearly replaced by new, larger molecules during subsequent passages. This phenomenon was observed in line 2 of *N. rustica* (Fig. 1B and C, 23°C-5-2), line 3 of sweet pepper (Fig. 1B and C, Pe-3), and line 3 of *N. benthamiana* (Fig. 1B and C, Be-3).

Doublets of full-length L RNA or DI RNA bands (Fig. 1A, To and Pe) were observed in Northern blot analysis. They could not be observed in all RNA preparations, although identical protocols for RNA extraction and electrophoresis were used. Using strand-specific riboprobes, double L and DI RNAs bands were shown to consist of viral and vc strands of TSWV (data not shown). These results confirmed earlier reports of differential strand migration of TSWV L and S RNAs (16).

Effect of inoculum concentration on the generation of DI RNAs. To analyze the effect of virus concentrations in the inocula on the generation of DI RNAs, plants of *N. rustica* were inoculated with a 5, 50, or 1,000 times diluted extract of wild-type TSWV-infected plants and, subsequently, serially passaged.

DI RNAs could not be detected in any of the lines after the fifth passage, when total RNA was analyzed by Northern blot hybridization (Fig. 1A, 23°C-5, -50, and -1,000). However, after the tenth passage (Fig. 1B), DI RNAs were found in two out of three lines inoculated with a five times diluted extract (23°C-5-2 and -3) and in one out of three lines with the 50 times diluted extract (23°C-50-1).

After the fifteenth passage (Fig. 1C), none of the lines inoculated with 1,000 times diluted extract contained DI RNAs, whereas they were present in two lines inoculated with five times diluted (23°C-5-2 and -3) and in all three lines with 50 times diluted extracts. Our results supported the general view (25,26) that the use of high virus concentration in the inocula promotes DI RNA generation.

Fig. 1. Northern blot hybridization of total RNA extracts from serially passaged tomato spotted wilt virus (TSWV)-infected plants collected during the **A**, fifth; **B**, tenth; and **C**, fifteenth passages using probes specific to the large RNA of TSWV. The generation of defective interfering (DI) RNAs of TSWV was tested in *Nicotiana rustica* inoculated with infected plant extracts diluted 5, 50, and 1,000 times (23°C-5, -50, and -1,000); in *N. rustica* incubated at 16, 23, and 30°C (16°C-5, 23°C-5, and 30°C-5); in the hosts *N. rustica* (23°C-5), tomato (To), sweet pepper (Pe), *N. benthamiana* (Be), *Datura stramonium* (Da), and *Emilia sonchifolia* (E); and in *N. rustica* inoculated with extracts from plants grown for 8 (23°C-5), 16 (I-16 [I = host inoculation]), and 32 (I-32) days postinoculation. Positions and sizes (kb) of large, medium, and small RNAs are shown. Three independent lines were tested for each tested condition (1, 2, and 3). **B**, Results of Northern blot hybridization on the ninth passage for I-32 (asterisk). nt = not tested.

Effect of temperature on the generation of DI RNAs. The influence of temperature on the generation of DI RNA molecules was also analyzed. *N. rustica* plants were serially inoculated with a five times diluted infected plant extract and incubated at 16, 23, or 30°C. Total RNA was extracted from plants after the fifth passage (Fig. 1A) and subjected to Northern blot analysis. The results showed that DI RNAs were generated in all three lines kept at 16°C (16°C), but in none of the lines kept at 23°C (23°C-5) and only in one line kept at 30°C (30°C-2). After the fifteenth passage, DI RNAs were found in two out of three lines incubated at 23°C (Fig. 1C, 23°C-5-2 and -3) and 30°C (Fig. 1C, 30°C-2 and -3).

Northern blot analysis of all individual passages demonstrated that the DI RNAs could already be detected in the second or third passage in the lines incubated at 16°C (Fig. 3), whereas the first DI RNA was detected in the fifth passage in a line kept at 30°C (data not shown). These results indicated that lower temperatures had a stimulating effect on the generation or accumulation of TSWV DI RNAs.

To confirm the importance of low temperature incubation on DI RNA formation and accumulation, their effect was studied in another series of three lines for only five passages. Northern blot analysis of the plants (Fig. 4) showed that all lines kept at 16 and 23°C (16°C-5 and 23°C-5) contained DI RNAs, while a DI RNA was present in only one of the three lines kept at 30°C (30°C-1). Analysis of the first to fourth passage plants incubated at 16°C revealed that the first DI RNAs could already be detected in the second or third passage, whereas they appeared after the third passage in the 23 or 30°C lines (data not shown). These results strongly suggested that low temperature consistently favors the generation and accumulation of TSWV DI RNAs in *N. rustica* plants.

Effect of inoculum concentration on the generation of DI RNAs in plants incubated at 16°C. The results presented showed that low temperature and high virus concentrations in the inocula accelerated the generation of DI RNAs in serially TSWV-inoculated plants. To study whether low temperatures can further influence the rate of the formation of DI RNAs when plants are inoculated with different virus concentrations in inocula, *N. rustica* plants were inoculated with 5, 50, or 1,000 times diluted extracts from infected plants, incubated at 16°C, and serially passaged five times. After these passages, no distinct effect of the different virus concentrations could be observed on the generation of DI RNAs (Fig. 4, 16°C-5, -50, and -1,000). DI RNAs were generated in all three lines incubated at 16°C, independently of the virus concentration in the inocula used. More detailed analysis demonstrated that the first DI RNAs could be detected after the first passage and the last ones in the fourth passage (data not shown).

Fig. 2. A, Northern blot hybridization of total RNA extracts after the fifth passage of sweet pepper lines 1 and 2 (Pe-1 and Pe-2) and *Datura stramonium* line 1 (Da-1). Hybridization was performed using probes specific to large RNA. Positions and sizes (kb) of large, medium, and small RNAs are shown. **B,** Electrophoresis of polymerase chain reaction-amplified DNA fragments from Pe-1, Pe-2, and Da-1. DNA size marker is indicated on the right (kb).

The generation of DI RNAs in *E. sonchifolia* at 16, 23, or 30°C. Since no DI RNAs were detected in *E. sonchifolia* plants incubated at 23°C, it was questioned whether other incubation temperatures could promote the generation of DI RNAs in plants of this species. The occurrence of DI RNAs was studied in serially inoculated *E. sonchifolia* plants incubated at 16, 23, or 30°C and passaged five times using two times diluted infected *E. sonchifolia* plant extracts. Northern blot analysis of samples collected after the fifth passage revealed that DI RNAs were completely absent in these lines (data not shown).

Effect of infection age on the generation of DI RNAs. To determine whether the length of infection would affect the generation of DI RNAs, inocula were prepared from *N. rustica* plants infected for 8, 16, or 32 days. Up to 15 serial passages were performed for the lines inoculated in 8- and 16-day intervals, whereas a total of nine passages were performed for plants infected for 32 days. After the fifth passage, no DI RNAs were detected in any of the three lines serially passaged at 8-day intervals (Fig. 1A, 23°C-5), while DI RNAs were observed in two of the three lines after 10 and 15 passages (Fig. 1B and C, 23°C-5-2 and -3). In the treatment in which the inoculum was prepared 16 days after inoculation, DI RNAs were observed in one line after the fifth passage (Fig. 1A, inoculation [I] 16-1), in two lines after the tenth passage (Fig. 1B, I-16-1 and -3), and finally in all three lines after the fifteenth passage (Fig. 1C). In lines inoculated with leaf extracts from plants incubated for 32 days, no DI RNAs were seen after the fifth passage (Fig. 1A, I-32), but two lines revealed the presence of DI RNAs in the total RNA extracts after the ninth passage (Fig. 1B, I-32-1). These results showed that the age of the infection in

Fig. 3. Hybridization of total RNA extracts from *Nicotiana rustica* plants in which tomato spotted wilt virus (TSWV) was passaged one to five times (lanes 1 to 5) in three lines kept at 16°C (16-1, 16-2, and 16-3) using large RNA-specific probes. Positions and sizes (kb) of the large, medium, and small RNA are shown. Defective interfering RNAs were detected at passage 2 in line 16-1 and 16-3, and at passage 3 in line 16-2.

Fig. 4. Northern blot hybridization of total RNA extracts prepared from plants after the fifth passage using probes specific to the large RNA. *Nicotiana rustica* plants were inoculated with five times diluted extracts from infected plants and incubated at different temperatures (16°C-5, 23°C-5, and 30°C-5), or inoculated with different inoculum concentrations (16°C-5, -50, and -1,000) and incubated at the temperature of 16°C. Positions and sizes (kb) of large, medium, and small RNAs are shown. Three independent lines were tested for each tested condition (1, 2, and 3).

plants from which the inocula were derived had no significant stimulating effect on favoring the generation of DI RNA in *N. rustica* plants.

DISCUSSION

In this report, the effects of hosts, virus concentrations in inocula, incubation temperatures, and ages of the infection in the inoculated plants were examined for their roles in the generation and accumulation of TSWV DI RNA molecules. A delay in symptom expression and the appearance of milder symptoms were usually associated with the emergence of DI RNAs in the TSWV isolate used, although the level at which the symptoms were attenuated varied with the DI RNA (data not shown). The symptom-attenuating property, the amplification by PCR using primers directed to both ends of the L RNA genome, and the large amount of smaller RNA molecules produced compared with the amount of L RNA indicated that the isolates consisted of genuine DI RNA molecules (12) of TSWV. A single DI RNA species usually dominated an isolate after a few rounds of serial inoculations. This was also observed after simultaneous inoculation of distinct DI RNAs in a single plant (data not shown).

DI RNAs could be readily detected after serial passages of TSWV, particularly in plants incubated at 16°C. DI RNA molecules could be detected as early as in the first passage, although they were usually found after the second passage. The more frequent generation of DI RNAs at 16°C could be due to changes in the RNA (or ribonucleoprotein complex) configurations leading to a lower fidelity of replication. Furthermore, the viral polymerase may perform suboptimally at lower temperatures, resulting in the formation of aberrant replication products. Most of the hitherto suggested models for the genesis of DI RNAs involve transcription or replication errors during viral RNA synthesis (17,20,25). Alternatively, this could also be the result of a change in the rate at which the various DI RNA molecules replicated. In soilborne wheat mosaic furovirus, higher temperatures increased the formation of shortened forms of RNA 2 (6), consequently showing an effect of temperature that was opposite to our observation. The importance of temperature on DI RNA generation was confirmed on TSWV, though its actual effect in the mechanism of RNA deletion remains unknown.

The formation and accumulation of DI RNAs is probably regulated by numerous factors that depend on the virus itself, the host, and the interaction between the virus and the host given a specific environmental condition. DI RNAs of CNV generated in *Nicotiana* species were effectively filtered out when this isolate was inoculated to cucumber plants (5), suggesting a strong influence of the host in the accumulation of DI RNAs. Our studies revealed a different type of effect of host from *E. sonchifolia* plants. The replication of DI RNAs was supported in *E. sonchifolia* plants when they were present in the inoculum (data not shown), although new DI RNAs could not accumulate in these plants under all conditions tested. The failure of this species to support the accumulation and generation of DI RNAs cannot be explained by a lower virus concentration in the inocula, as the used inocula were adjusted to provide comparable virus concentrations to those used for the other plants. Our results showed that *E. sonchifolia* is potentially a useful host to maintain isolates of TSWV free of DI RNAs.

In addition to the effects of temperature and host, high virus concentrations were also found to favor the formation of TSWV DI RNAs. The use of inocula prepared from extracts diluted 50 times, or less, resulted in the formation of DI RNAs, whereas none were found when 1,000 times diluted extracts were incubated at 23°C. These results confirmed previous studies showing that DI RNAs were readily generated when serially inoculated with virus inocula in high concentrations (4,14,22). The use of high virus concentrations increases the likelihood of coinoculating the initially generated DI RNA with helper genomic RNAs (24).

After 15 serial inoculations, several of the lines were subjected to Northern blot analysis for detection of defective RNA molecules originating from M and S RNAs. However, no such defective RNA molecules were found (data not shown). This observation confirmed earlier reports (22,23) that TSWV DI RNAs are only generated from the L RNA.

This study had a primary aim to determine some of the factors that could affect the generation of TSWV DI RNAs upon inoculation of plants with TSWV. The results showed that to secure or maintain the original wild-type form of a TSWV isolate plants have to be inoculated with a highly diluted plant extract, plants have to be grown at higher temperatures, *E. sonchifolia* has to be used as the maintenance host, and, most importantly, the TSWV isolate must not be passaged serially frequently. Instead of avoiding the formation of DI RNA, omission of these procedures leads to the production of a large range of different DI RNAs that, in turn, will enable us to study several aspects of viral replication, packaging, and evolution.

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