Synergistic Interaction Between *Tomato chlorosis virus* and *Tomato spotted wilt virus* Results in Breakdown of Resistance in Tomato

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ABSTRACT

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Multiple viral infections frequently are found in single plants of cultivated and wild hosts in nature, with unpredictable pathological consequences. Synergistic reactions were observed in mixed infections in tomato plants doubly infected with the positive-sense and phloem-limited single-stranded RNA (ssRNA) crinivirus *Tomato chlorosis virus* (ToCV) and the negative-sense ssRNA tospovirus *Tomato spotted wilt virus* (TSWV).

Synergism in a tomato cultivar susceptible to both viruses resulted in a rapid death of plants. A pronounced enhancement of ToCV accumulation mediated by TSWV co-infection was observed with no evident egress of ToCV from phloem tissues. No consistent alteration of TSWV accumulation was detected. More remarkable was the synergism observed in tomato cultivars which carry the *Sw-5* resistance gene, which are resistant to TSWV. Pre-infection with ToCV resulted in susceptibility to TSWV, whereas co-inoculations did not. This suggested that a threshold level or a time lapse is needed for ToCV to interfere or downregulate the defense response in the TSWV-resistant plants.

Multiple viral infections that may result in synergisms or antagonisms frequently are found in nature, with unpredictable pathological consequences. Synergistic interactions resulting from mixed infections with two or more viruses are common and well documented in plants. In most cases, they result in an increase in the titers of one or both viruses and symptoms may be enhanced (18,25,30,37). Classical examples are the synergisms between potyviruses (family *Potyviridae*) and luteoviruses (family *Luteoviridae*) (2) or potexviruses (family *Potexviridae*) (25,27,37). Although many studies on synergism between plant viruses have been described, the potyvirus-associated synergistic interactions are the best studied. In this case, a dramatic increase of disease symptoms usually is observed in doubly infected plants which often corresponds to an increase in virus titers of the beneficiary virus, whereas the potyvirus accumulation is not affected (6,15,37). Synergistic interactions also can result in an increase in the titers of both viruses, as reported in mixed infections of the tritrovirus *Wheat streak mosaic virus* (family *Potyviridae*) and the machlomovirus *Maize chlorotic mottle virus* (family *Tombusviridae*) in corn (31). Even more complex viral interactions also have been detected, such as the triple synergism between the potyvirus *Beet mosaic virus*, the closterovirus *Beet yellows virus* (family *Closteroviridae*), and the polerovirus *Beet western yellows virus* (BWYV, family *Luteoviridae*) in sugar beet. In this case, a pronounced effect on stunting severity in triply infected sugar beet plants was observed, correspondent with an increase of titers of all three viruses (42). Viral synergism could affect various growth parameters such as plant height, weight, and yield, and can be expressed by degrees of symptoms severity (21). In extreme cases, viral synergy leads to plant death.

The mechanisms and viral products involved in viral synergistic interactions remain unresolved in most cases. As mentioned, the best characterized are the synergisms involving potyviruses. Pruss et al. (25) demonstrated that the potyvirus *Tobacco etch virus* genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses such as *Cucumber mosaic virus* (CMV, family *Bromoviridae*, genus *Cucumovirus*) and *Tobacco mosaic virus* (TMV, genus *Tobamovirus*). Further research on this interaction has indicated the viral RNA silencing suppressor protein P1/HC-Pro to be involved in the synergistic effect, possibly by decreasing the targeting of the other virus by the RNA silencing machinery (38,39). Other recent reports trying to elucidate the mechanisms involved in synergisms suggested that virus-virus interactions in mixed infection might be a result of a complementation of a movement deficiency by one of the viruses (29,30). An interesting synergistic interaction was described between the potyvirus *Sweet potato feather mottle virus* (SPFMV) and the crinivirus *Sweet potato chlorotic stunt virus* (SPCSV, family *Closteroviridae*) causing a novel sweet potato virus disease (SPVD), characterized by the potyvirus-resistance breakdown in doubly infected sweet potato plants (18). The SPVD seems to be an unusual synergistic interaction in that the potyvirus component is not the cause of synergism but the beneficiary by enhancement of SPFMV multiplication by the phloem-limited crinivirus.

Tomato (*Solanum lycopersicum* L.) is a crop severely affected by several viral diseases, in which mixed viral infections may result in synergisms or antagonisms that could compromise virus control. The phloem-limited crinivirus *Tomato chlorosis virus* (ToCV) was discovered initially in diseased tomato and has been identified as a serious problem for tomato production in many parts of the world, particularly in the United States, Europe, and Asia, although there is little information available on crop losses caused (11,44). The appearance of ToCV epidemics in tomato fields in southern Spain has been alarming. The outbreaks were

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associated with high populations of *Bemisia tabaci* Gennadius (*Hemiptera: Aleyrodidae*), and incidences of more than 30% of symptomatic plants in individual fields were common (22,23). The severity of symptoms and damage vary depending on the cultivar but, frequently, tomato plants infected with ToCV showed an irregular distribution of symptoms (22,44). Symptoms caused by ToCV, like those from other members of the family *Clustero-viridae*, frequently are misattributed to abiotic disorders, such as physiological or nutritional disorders, or even to phytotoxicity caused by chemicals used for plant protection. The viral genome recently has been characterized and revealed a typical crinivirus genome structure (19,43).

The tospovirus *Tomato spotted wilt virus* (TSWV) (family *Bunyaviridae*) is another important plant-infecting virus that causes severe damage to tomato and other crops. TSWV has been reported to cause significant yield losses in all major tomato-growing areas around the world. This virus is transmitted by thrips species and is one of the most widely spread plant virus, infecting hundreds of plant species (14,24). Cultural practices have been only marginally effective in controlling TSWV. However, introgression of natural resistance from wild relatives has resulted in commercial tomato cultivars with broad resistance to different tospoviruses due to the presence of the *Sw-5* resistance gene (3,13,32,33). These cultivars are widely cultivated commercially and limit TSWV damage.

The simultaneous presence of high populations of thrips and whiteflies in tomato fields of many tropical and subtropical regions worldwide and their efficiency to transmit tospoviruses and criniviruses suggests that ToCV-TSWV mixed infections can occur where these viruses are present. However, to date, little is known about synergistic interactions between viruses in tomato, and how viral mixed infections could affect plant physiology and host defense responses such as expression and stability of resistance genes.

The aim of this report is to study the synergistic interaction between the positive-sense and phloem-limited single-stranded RNA (ssRNA) virus ToCV and the negative-sense ssRNA virus TSWV in tomato. A dramatic increase in symptom severity was observed in plants of a susceptible cultivar after simultaneous inoculation of both viruses, leading to a rapid death of plants. Strikingly, pre-infection with ToCV of tomato cultivars resistant to TSWV resulted in loss of TSWV resistance.

MATERIALS AND METHODS

Tomato cultivars and virus inoculations. Tomato cvs. Moneymaker (susceptible to both ToCV and TSWV) and Anastasia F₁ (Seminis Vegetable Seeds Iberica, Almería, Spain; susceptible to ToCV and resistant to TSWV) were used for single and mixed inoculation with TSWV and ToCV. Tomato cvs.

TABLE 1. Inoculation treatments (virus combination) of tomato plants (cvs. Moneymaker and Anastasia) singly or mixed inoculated with *Tomato chlorosis virus* (ToCV) and *Tomato spotted wilt virus* (TSWV) in a simultaneous (co-inoculation) or delayed inoculation format

Treatments	Time 1	Interval (days) ^w	Time 2
1	ToCV	10	TSWV
2	TSWV	10	ToCV
3	ToCV + TSWV	SI	— ^x
4	ToCV	10	Mock ^y
5	TSWV	10	Mock ^z
6	Mock ^y	10	ToCV
7	Mock ^z	10	TSWV
8	Mock ^z	10	Mock ^y

^w SI = simultaneous inoculation.

^x No inoculation.

^y Mock-inoculated using TSWV inoculation buffers.

^z Mock-inoculated using ToCV-free whiteflies.

DRWV 7456 (De Ruiter Seeds, Almería, Spain) and Vinchy RZ (Rijk Zwaan Iberica, Almería, Spain) also were used in experiments to test the effect of viral synergism in other TSWV-resistant cultivars. ToCV isolate AT80/99 (19) was obtained from a naturally infected tomato plant from Málaga (southern Spain) and was maintained at the Estación Experimental La Mayora-CSIC in tomato cv. Moneymaker by grafting and periodic transmission using *B. tabaci* (Q biotype). TSWV isolate TM1 (26) was obtained from a naturally infected tomato plant from Barcelona (northeastern Spain) and has been maintained in the same research station by storage of freshly infected tissues at -80°C . The TSWV isolate was multiplied by mechanical inoculation (described below) of *Nicotiana rustica* L. plants, from which systemically infected leaves were used as fresh inoculum sources in the assays.

Tomato test plants were mechanically inoculated with TSWV using extracts from freshly infected *N. rustica* leaves obtained at 1:10 (wt/vol) dilution ratio in 10 mM phosphate buffer, pH 7.0, containing 10 mM Na₂SO₃. The first (Table 1, time 1) or third (Table 1, time 2) leaf above the cotyledons of plants at three- or five-leaf growth stages, respectively, were inoculated. ToCV was inoculated in the leaf opposite to the one inoculated with TSWV with 20 viruliferous whiteflies per plant applied using a clip-on cage (22). Prior to inoculation, whiteflies were allowed to feed for 48 h on ToCV-infected tomato plants for virus acquisition and subsequently transferred to test plants for a 48-h inoculation access period. Whiteflies were eliminated after this period by spraying the plants with imidacloprid (Confidor; Bayer A.G., Leverkusen, Germany). Eight treatments (virus combinations) (Table 1) with the two tomato cvs. Moneymaker and Anastasia were assayed. Ten plants were inoculated per treatment in a simultaneous (co-inoculation) or delayed (10-day interval) inoculation format. Mock inoculations were performed with virus-free whiteflies and inoculation buffer as ToCV- and TSWV-negative controls, respectively. After inoculation, plants were maintained in a greenhouse at 24°C with a light-dark cycle of 16 and 8 h, respectively, and kept growing for 50 days. Virus symptoms were recorded at 8, 15, 20, 25, and 30 days postinoculation (dpi) (counted after the inoculation of the second virus in the case of delayed inoculations) and, when needed, plant growth was estimated from shoot fresh weight at 30 dpi. The entire experiment was repeated two times over a period of 12 months. Inoculation of plants of cvs. DRWV 7456 and Vinchy RZ together with those of the corresponding control cv. Anastasia followed the same delayed-inoculation scheme of treatments 1 and 7 (Table 1).

Determination of TSWV and ToCV levels in infected tomato plants. Estimation of TSWV accumulation was assessed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (8) using polyclonal antisera against the N protein obtained from Loewe Biochemica GmbH (Otterfing, Germany). ELISA was performed according to Avila et al. (1) using dilutions recommended by the supplier for anti-TSWV immunoglobulin G and for anti-TSWV alkaline phosphatase conjugate antisera. From all treatments (Table 1, virus combinations), one young terminal leaf of each of five randomly selected plants was collected at 8, 15, 20, and 25 dpi, weighed, and ground in the ELISA sample buffer at 1:10 (wt/vol) dilution ratio. Then, dilutions of 1:100 and 1:1,000 were obtained from each plant extract. Sample aliquots of 150 µl of each plant extract dilution (1:10, 1:100, and 1:1,000) then were applied to a microtiter plate. ELISA absorbance values (at 405 nm) were recorded 1 h after substrate addition using an Anthos 2001 microplate reader (Anthos Labtec Instruments GmbH, Salzburg, Austria). In every case, ELISA values used for estimation of TSWV levels were those from a dilution that fell within the linear range of the relationship between dilution and absorbance measure. Treatment means of virus accumulation were compared with a Tukey b test following analysis of variance (SPSS for Windows statistical software, release 12.0; SPSS Inc., Chicago).

For estimation of ToCV levels, samples from inoculated plants were collected at 8, 15, 20, and 25 dpi and analyzed by dot blot hybridization. Samples consisted of one young terminal leaf of each of five randomly selected plants. Total nucleic acid (TNA) extracts were prepared as reported by Celix et al. (7), except that TNA finally were dissolved in 50 μ l of sterile H₂O. A dilution series of TNA (2 μ l/sample at 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32) was applied to positively charged nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany) and hybridized with a ToCV-specific probe. For this, a digoxigenin (DIG)-11-UTP-labeled RNA probe to detect the positive-sense ToCV RNA was synthesized by in vitro transcription from a clone (pToCV-22) which contains the coat protein gene derived for the isolate AT80/99 of ToCV from Spain (19). The clone was linearized with *Sa*I for transcription of negative-sense RNA using T7 RNA polymerase (Roche Diagnostics). Synthesis of ToCV DIG-labeled RNA probe, blot hybridization conditions, and chemiluminescent detection were performed using reagents supplied with the DIG-labeling and detection kit (Roche Diagnostics) and according to manufacturer's protocols. Assessment of ToCV RNA content in each tested sample was done by densitometric measure of hybridization signals obtained in autoradiographs. Plant ribosomal RNA concentrations (measured using a DIG-labeled DNA-probe prepared by random priming to a 18S ribosomal RNA gene clone kindly provided by E. R. Rodríguez-Cerezo, CNB-CSIC, Madrid, Spain) were used as an internal standard to equilibrate TNA loading among samples. Densitometry measurements were expressed in pixels measured by an image analysis system (EDAS 120 Image Analysis System; Eastman Kodak, Rochester, NY). In every case, the densitometry values used for estimation of ToCV levels were those from a dilution that fell within the linear range of the relationship between dilution and densitometry measure. Distribution of values were represented by Box-and-Whisker plots (36) and pairwise comparisons between treatments were

made with the nonparametric U-Mann-Whitney's test (SPSS for Windows statistical software; release 12.0; SPSS Inc.).

Cytological detection of viral RNA by in situ hybridization. In situ hybridization was performed in systemically infected leaves of singly and doubly infected cv. Moneymaker plants. One upper leaf per plant was detached from the stem at 15 dpi and used for preparing tissue sections for hybridization. ToCV (see above) and TSWV (based on a TSWV NSs gene cDNA clone) DIG-labeled RNA transcription probes made to detect positive-sense RNAs were used for hybridization. Probes were hybridized to tissue sections of 15 μ m and detected using an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics); 5-bromo-4-chloro-3-indoylphosphate and nitro-blue tetrazolium were used as chromogenic substrates (40,41). Plant tissues were fixed in 4% formaldehyde and prepared for in situ hybridization according to Jackson (17). Processed sections were mounted in Shandon Mount Toluene Base (Shandon, Pittsburgh) with a prior counterstaining in 0.1% calcofluor white M2R (Sigma-Aldrich, St. Louis) and photographed using transmitted light microscopy or combined epifluorescence (UV; calcofluor staining of cell walls) and transmitted light microscopy. Mock-inoculated plants were used as negative controls.

RESULTS

Viral synergism in co-inoculated cv. Moneymaker plants.

The TSWV and ToCV isolates used in this study did infect Moneymaker plants systemically. Plants singly infected with TSWV (Table 1, treatment 5 and 7) developed TSWV symptoms starting at 8 dpi that, in our conditions consisted, of epinasty, leaf deformation, and discoloration of leaves; plant growth was reduced compared with the mock-inoculated plants (Fig. 1A). In plants singly infected with ToCV (Table 1, treatments 4 and 6), development was similar to that of the mock-inoculated plants, and yellowing symptoms in lower leaves typical of tomato chlorosis disease were not observed in the period analyzed (Fig. 1A, compare ToCV and Mock). Plants of treatments 1 and 2 in which inoculation of TSWV and ToCV were delayed exhibited symptoms that did not differ from those observed in control plants singly infected with TSWV (data not shown). Tomato plants co-inoculated with TSWV and ToCV (Table 1, treatment 3) showed significant viral synergism, with a dramatic increase in the severity of disease symptom (Fig. 2) beginning soon after co-infection

Fig. 1. Virus symptoms observed for infections with *Tomato spotted wilt virus* (TSWV), *Tomato chlorosis virus* (ToCV), or co-infections of TSWV and ToCV in susceptible tomato cv. Moneymaker plants. **A**, Tomato plants singly infected with ToCV, co-infected with ToCV and TSWV, singly infected with TSWV, and mock inoculated at 20 days postinoculation (dpi). **B**, Detail of doubly infected plants at 20 dpi showing lower leaves with severe yellowing and red and brown flecks and younger leaves exhibiting severe mosaic and wilting. **C**, Symptoms in co-infected plants evolved to a pronounced necrosis of leaves and stems leading to plant death at 30 dpi.

Fig. 2. Severity of symptoms (from 0, absence, to 5, maximum severity) observed at different times postinoculation in tomato cv. Moneymaker plants infected only with *Tomato chlorosis virus* (ToCV) or *Tomato spotted wilt virus* (TSWV), co-infected with both viruses (ToCV+TSWV), or mock inoculated. Mean $\pm$ standard error values from 10 plants per treatment are indicated.

compared with singly inoculated plants (Fig. 1A and B, symptoms at 20 dpi). Plant growth was severely reduced after co-infection. The lower leaves developed severe yellowing followed by development of red and brown flecks, and they collapsed. The younger leaves exhibited severe mosaic and wilting by 20 dpi. Symptoms in co-inoculated plants evolved to a pronounced necro-

TABLE 2. Plant growth estimated from shoot fresh weight of tomato plants mock-inoculated (Mock), singly inoculated with *Tomato spotted wilt virus* (TSWV) or *Tomato chlorosis virus* (ToCV), or co-inoculated with ToCV and TSWV (ToCV+TSWV), at 30 days postinoculation

Treatment	Fresh weight (g) ^z
ToCV+TSWV	8.76 ± 0.34 c
TSWV	16.07 ± 3.37 b
ToCV	28.35 ± 1.22 a
Mock	27.28 ± 3.41 a

^z Mean ± standard error values from 10 plants per treatment are shown in each case. Values with the same letter are not significantly different (Tukey b test, *P* = 0.05).

Fig. 3. Accumulation of *Tomato chlorosis virus* (ToCV) at 8, 15, 20, and 25 days postinoculation in young leaves of tomato cv. Moneymaker plants infected only with *Tomato chlorosis virus* (ToCV) or co-infected with ToCV and *Tomato spotted wilt virus* (TSWV). Virus content is estimated from densitometry measure of hybridization signals obtained in digitized imaging of autoradiographs (kilopixels) of dot blot hybridizations with a ToCV-specific probe of total nucleic acids extracted from plants and normalization of values per plant with the signal from the hybridization with a 18S ribosomal RNA probe. Densitometry values corresponding to the dilution 1:1 of total nucleic acids are represented in a Box-and-Whisker plot. For each date, values with the same letter are not significantly different (nonparametric U-Mann-Whitney's tests, *P* = 0.05).

TABLE 3. Accumulation of *Tomato spotted wilt virus* (TSWV) estimated by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) from tomato plants mock-inoculated (Mock), singly inoculated with TSWV, or co-inoculated with TSWV and *Tomato chlorosis virus* (ToCV+TSWV)

Treatment	Absorbance value at 405 nm ^z			
	8 dpi	15 dpi	20 dpi	25 dpi
ToCV+TSWV	0.416 ± 0.030 a	0.735 ± 0.046 a	0.676 ± 0.035 a	0.808 ± 0.016 b
TSWV	0.514 ± 0.039 a	0.456 ± 0.024 b	0.771 ± 0.044 a	1.124 ± 0.077 a
Mock	0.091 ± 0.011 b	0.040 ± 0.002 c	0.090 ± 0.008 b	0.188 ± 0.004 c

^z Mean ± standard error DAS-ELISA absorbance value at 405 nm 1 h after addition of the substrate for the dilution 1:100 (wt/vol) of extracts obtained from young leaves of five randomly selected plants per treatment tested at 8, 15, 20, and 25 days postinoculation (dpi). For each date analysis, absorbance values with the same letter are not significantly different (Tukey b test, *P* = 0.05). Mock-inoculated tomato plants were used as negative controls.

sis of leaves and stems and all doubly infected plants died at 30 dpi (Fig. 1C). Plant growth in the treatments was estimated from shoot fresh weight at 30 dpi. TSWV+ToCV co-infected plants had two to three times less plant biomass accumulation compared with singly or mock-inoculated plants (Table 2).

Increased ToCV accumulation in co-inoculated cv. Moneymaker plants. DAS-ELISA (TSWV) and dot blot hybridization (ToCV) were used to estimate virus accumulation in singly and doubly infected Moneymaker plants. Similar results were obtained in two independent experiments. In the case of ToCV, a significantly higher ToCV accumulation was observed in TSWV+ToCV-infected plants compared with plants singly infected with ToCV (Fig. 3) except for 8 dpi. Maximum ToCV accumulation differences occurred at 20 dpi (Fig. 3). In contrast, TSWV accumulation did not show a consistent tendency when singly and doubly inoculated plants were compared. Either no significant differences were observed or slightly higher or lower virus titers were detected in doubly inoculated plants compared with singly inoculated ones (Table 3).

Confinement of ToCV to phloem tissues of cv. Moneymaker plants. A dramatic increase in ToCV accumulation was observed in leaf tissues of tomato cv. Moneymaker plants co-infected with TSWV; therefore, the distribution of ToCV in systemically infected leaves of singly and doubly infected plants was studied by in situ RNA hybridization. In all samples analyzed, ToCV RNA exhibited a similar confinement to phloem cells in either singly or doubly infected plants (Fig. 4B and C, ToCV+TSWV-infected plants). No difference in TSWV distribution in plant tissues was observed between singly or doubly infected plants (data not shown).

Resistance breakdown in TSWV-resistant tomato plants. The TSWV-resistant Anastasia plants inoculated with ToCV either singly or doubly inoculated with TSWV exhibited 100% infection with ToCV. Typical symptoms of interveinal yellowing (22,23) on the lower leaves were observed at 30 dpi and virus could be detected readily in the young noninoculated upper leaves. Anastasia plants inoculated with TSWV only or simultaneously inoculated with TSWV and ToCV showed neither TSWV-associated symptoms nor TSWV accumulation in young noninoculated leaves at any time analyzed postinoculation. However, in Anastasia plants inoculated first with ToCV and 10 days later with TSWV (Table 1, treatment 1), typical TSWV symptoms were observed and TSWV was detected in a number of them (4 and 8 of 10 plants inoculated in two independent experiments), indicating a breakdown of the TSWV resistance. Plant growth was reduced and leaf deformation was apparent in those plants, comparable to symptoms induced by TSWV in susceptible tomato plants (Fig. 5A). Chlorotic spots that turned into necrotic spots were observed in systemically infected leaves by 20 days post-TSWV inoculation (Fig. 5B). General chlorosis, deformation, and veinal necrosis were observed in the leaves and necrosis in the stems by 30 days after TSWV inoculation (Fig. 5C). In order to test whether or not breakdown of resistance also occurred in other commercial TSWV-resistant tomato lines, cvs. DRWV 7456 and Vinchy RZ were tested. The loss of TSWV-resistance was confirmed (Table 4). It should be noted that, in this experiment,

breakdown of resistance manifested as chlorosis and deformation in noninoculated leaves of DRW 7456 plants that gave a positive reaction in ELISA; however, in Anastasia and Vinchy RZ plants, leaf petiole and stem necrosis occurred but no positive reaction was detected in ELISA tests of young noninoculated leaves (Table

4). Also, few plants inoculated only with TSWV exhibited leaf petioles and stem necrosis (Table 4, cvs. Anastasia and Vinchy RZ) and leaf deformation (cv. DRWV 7456). However, the number of plants showing TSWV-like symptoms was remarkably higher in the doubly inoculated plants.

DISCUSSION

TSWV and ToCV are fundamentally different viruses. TSWV is a negative-sense ssRNA virus and ToCV is a phloem-limited positive-sense RNA virus. These viruses are very efficiently transmitted by thrips and whiteflies, respectively. High populations of thrips and whiteflies often are found co-infesting tomato fields worldwide. Therefore, mixed infections of TSWV and ToCV are predicted to be common in crops in areas where these two viruses are present, with unpredictable pathological consequences. Here, we report that, in tomato, a striking synergistic interaction was found between ToCV and TSWV, resulting in an overexpression of virus disease symptoms accompanied by increased ToCV titers in a susceptible tomato and a loss of virus resistance in TSWV-resistant tomato cultivars.

A dramatic increase of disease symptom severity that led to a rapid death of plants was observed after co-inoculation of ToCV and TSWV in plants of Moneymaker, a tomato cultivar susceptible to both viruses. This synergistic interaction resulted in an exacerbation of ToCV-associated symptoms, probably because of the observed stimulation of ToCV accumulation in leaf tissues in the presence of TSWV during the course of co-infection. The remarkable increase of ToCV accumulation hinted at a possible new ability acquired by ToCV to egress the phloem and infect mesophyll cells during co-infection with TSWV, as observed in other synergistic interactions (2). However, in situ hybridization

Fig. 4. In situ localization of *Tomato chlorosis virus* (ToCV) nucleic acids in phloem tissue. Transversal sections of leaf petiole tissues from tomato cv. Moneymaker plants **A**, mock inoculated or **B**, co-inoculated with ToCV and *Tomato spotted wilt virus*, and **C**, longitudinal sections of leaf blade tissues from co-inoculated plants; tissues were obtained from young noninoculated leaves at 15 days postinoculation and hybridized with a ToCV-specific digoxigenin-labeled RNA probe. Arrows show ToCV-specific hybridizations signals in the phloem. Sections were photographed under light microscope. X = xylem, Ph = phloem, ePh = external phloem, iPh = internal phloem, Par = parenchyma. Thickness of sections is 15 µm. Scale bars indicate 500 µm in **A** and **B** and 100 µm in **C**.

Fig. 5. Loss of *Tomato spotted wilt virus* (TSWV) resistance in tomato cv. Anastasia. Plants were inoculated with *Tomato chlorosis virus* (ToCV) only, inoculated first with ToCV and 10 days later with TSWV (ToCV+TSWV), or inoculated with TSWV alone (TSWV). **A**, A synergistic interaction resulting in TSWV-resistance breakdown is observed in the doubly infected tomato plants at 20 days post-TSWV inoculation. Details of **B**, the chlorotic spots in systemically infected leaves that turn into necrotic spots at 20 days post-TSWV inoculation and **C**, the severe TSWV symptoms characterized by general chlorosis and vein necrosis in young leaves and stems by 30 days after TSWV inoculation in doubly infected plants are shown.

studies did not confirm this hypothesis, and no alleviation of ToCV phloem confinement was observed. It is possible that the higher levels of ToCV during mixed infection with TSWV might reflect a more efficient ToCV accumulation in the phloem, as is the case for the synergism between the luteovirus BWYV and the potyvirus *Beet mosaic virus* in sugar beet (42). In contrast, no consistent trend or alteration was observed for TSWV accumulation by co-infection with ToCV, suggesting a role for this virus similar to that of potyviruses in the majority of the synergistic interactions reported so far (38). Virus-enhanced accumulation that parallels the one described here also was observed in the monocotyledonous plant host barley (*Hordeum vulgare* L.) for TMV when co-inoculated with the hordeivirus *Barley stripe mosaic virus* (BSMV, genus *Hordeivirus*), with BSMV titer unaffected (10,16).

The combination of results from experiments in the susceptible tomato cv. Moneymaker suggested that the presence of TSWV in co-infected plants favors ToCV infection. Enhanced ToCV accumulation might occur because TSWV facilitates ToCV replication or movement or counteracts host defense responses against ToCV. In fact, most of the virus synergistic interactions reported so far are suggested to be the result of suppression of host defense mechanisms or complementation of a movement deficiency by one of the viruses in mixed infections (4,29,30,37,38). For example, synergism between potyviruses and the polerovirus *Potato leaf roll virus* (PLRV) (2,30) was suggested to be based, in part, on the potyvirus-silencing suppressor P1/HC-Pro (39) that facilitates the increased accumulation of the polerovirus in doubly infected plants without detectable egress from vascular tissues. The observations made in this report for ToCV and TSWV have parallels with those results. A similar interaction also was observed in a potyvirus–cucumovirus synergism in which the 2b protein, a known suppressor of RNA silencing (4) of CMV, seems to interfere in the counterdefense mechanism that restricts systemic spread of the potyvirus *Potato virus Y* (PVY) in tobacco (29). Moreover, a role in overriding putative host repressors in phloem tissues is suggested for some silencing suppressors, such as the P1/HC-Pro of potyviruses or the TrAP of geminiviruses (9,34,39), facilitating long-distance virus spread and accumulation in infected plants. In this sense, it is interesting to note that the NSs protein of TSWV recently was proved to be a strong silencing suppressor (5,35) and, therefore, it is an excellent candidate to be tested for its involvement in the ToCV accumulation enhancement, which merits further study.

Except for the treatment in which ToCV and TSWV were inoculated simultaneously, synergistic interaction was not observed in any other virus combination assayed in Moneymaker (Table 1). These results suggest that timing of virus onset or protein expression might be important to promote the synergism observed. Also, as demonstrated for the synergism between CMV and the potyvirus *Pepper mottle virus* in pepper, the developmental stage of the plant might affect viral titers, thereby influencing the level of synergism (21).

A synergistic interaction was shown in this research in the commercially grown tomato cv. Anastasia when challenged with the two viruses. Under normal conditions, this cultivar is susceptible to ToCV but resistant to TSWV. It was demonstrated that the presence of ToCV in Anastasia plants prior to TSWV inoculation compromised the TSWV resistance. Similar breakdown of TSWV resistance was not observed in plants inoculated with ToCV and TSWV simultaneously; therefore, a possible threshold titer of the former virus or level of ToCV proteins might be required to counteract the protection provided by the resistance gene. TSWV resistance breakdown also was true for TSWV-resistant commercial tomato cultivars tested other than Anastasia, such as DRW 7456 and Vinchy RZ. Resistance to TSWV of Anastasia, DRW 7456, and Vinchy RZ might be based on the *Sw-5* gene, because this is the resistance gene used commercially (24,32). In fact, presence of *Sw-5* was confirmed in these resistant tomato cultivars by PCR using primers designed from the sequence of this gene (32) (data not shown). Therefore, our results might suggest that ToCV could interfere with the *Sw-5*-resistance mechanisms independently of the genetic background of the tomato cultivar tested. Although *Sw-5* provides broad resistance to tospoviruses (3,33), sporadic infection by TSWV can be observed in *Sw-5*-tomato plants (28,33), which has been explained by incomplete *Sw-5* gene penetrance or by gene dosage effect derived from the variable allelic composition in the *Sw-5* gene cluster (3,32,33). Sporadic infection also was observed in our experiments in few Anastasia, DRWV 7456, and Vinchy RZ tomato plants singly inoculated with TSWV. The exact mechanism by which the *Sw-5* gene confers resistance to tospoviruses in tomato has not been elucidated (32), although it is expected to respond to a positive model of resistance because of the dominance of this gene (12). We can speculate that ToCV contains a repressor of the resistance response in resistant plants and, thus, may suppress the protection conferred by the resistance gene. Overcoming of host defenses also seems to be the case of the synergistic interaction causing SPVD that results in a potyvirus-resistance breakdown in doubly infected plants (18). In this case, the phloem-limited crinivirus enhances the potyvirus multiplication, possibly through interference of a systemic signaling required for resistance. In our case, further experiments using a system to express ToCV putative proteins in TSWV-resistant tomato plants previous to TSWV inoculation might provide insight into the basis of the synergistic interaction found. The presence of RNA silencing suppressors has been reported in closteroviruses (20); therefore, the possible involvement of this mechanism in the TSWV resistance breakdown should be taken into account.

The work reported here shows, for the first time, a synergistic interaction involving a tospovirus, and should be seen as an alert for tomato breeders and growers. Based on difficulties in identifying virus symptoms and crop losses caused by ToCV (19,22,44), infections with this virus frequently seem to be overlooked and infected plants are maintained in the field. Therefore, in regions in which ToCV and TSWV are present, tomato plants infected with

TABLE 4. *Tomato spotted wilt virus* (TSWV) infection and symptom development in plants of TSWV-resistant commercial tomato cultivars singly inoculated with TSWV or doubly inoculated with *Tomato chlorosis virus* (ToCV) and TSWV

Cultivar	No. of TSWV infected or symptomatic plants/no. plants inoculated ^z					
	TSWV		ToCV+TSWV		Mock	
	ELISA	Symptoms	ELISA	Symptoms	ELISA	Symptoms
DRWV 7456	1/5	1/5	3/10	3/10	0/5	0/5
Vinchy RZ	0/5	3/5	1/10	9/10	0/5	0/5
Anastasia	1/5	1/5	2/10	10/10	0/5	0/5

^z Plants were inoculated first (three-leaf growth stage) with ToCV and 10 days later with TSWV and evaluated 30 days later for symptom development and for presence of TSWV in young, noninoculated leaves. Virus detection was based on double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). Symptoms consisted of mosaic and leaf deformation in DRWV 7456 and of necrotic streaks followed by wilting in leaf petioles and stems in Vinchy RZ and Anastasia.

ToCV could serve as a background for TSWV mixed infections. Because double infections are likely to occur in tomato fields, ToCV-TSWV synergistic interaction could result in increased crop losses and even more drastic damage if breakdown of TSWV resistance occurs.

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