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Synergism and negative interference during co-infection of tomato and *Nicotiana benthamiana* with two bipartite begomoviruses

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ABSTRACT

In Brazil, at least eight begomoviruses including *Tomato rugose mosaic virus* (ToRMV) and *Tomato yellow spot virus* (ToYSV) infect tomatoes. ToYSV symptoms in tomato and *Nicotiana benthamiana* appear earlier and are more severe compared to those of ToRMV. We investigated the role of several factors in this differential adaptation. To analyze infection kinetics, a single leaf was inoculated and subsequently detached after different periods of time. Viral DNA accumulation was quantified in plants, viral replication was analyzed in protoplasts, and tissue tropism was determined by *in situ* hybridization. Results indicate that ToYSV establishes a systemic infection and reaches a higher concentration earlier than ToRMV in both hosts. ToRMV negatively interferes with ToYSV during the initial stages of infection, but once systemic infection is established this interference ceases. In *N. benthamiana*, ToYSV invades the mesophyll, while ToRMV is phloem-restricted. During dual infection in this host, ToYSV releases ToRMV from the phloem.

Introduction

The genus *Begomovirus* includes most of the economically important species of the *Geminiviridae* (Stanley et al., 2005). Begomoviruses have small, circular, single-stranded DNA genomes consisting of one or two components, each approximately 2600 nucleotides in length, encapsidated in twinned icosahedral particles (Rojas et al., 2005; Stanley et al., 2005). They are transmitted by the whitefly *Bemisia tabaci* (Homoptera: Aleyrodidae) and infect dicot species. Begomovirus diseases are a major factor limiting crop yields in tropical and subtropical regions (Briddon, 2003; Monci et al., 2002; Morales and Anderson, 2001; Ndunguru et al., 2005; Were et al., 2004).

In Brazil, tomatoes are infected by at least eight begomoviruses (Ribeiro et al., 2003). Some of these viruses are becoming prevalent in the major tomato-producing areas of the country, and mixed infections appear to be common in the field (Castillo-Urquiza et al., 2008; Fernandes et al., 2008). We have previously described two of these begomoviruses, *Tomato rugose mosaic virus* (ToRMV) (Fernandes et al., 2006) and *Tomato yellow spot virus* (ToYSV) (Calegario et al., 2007). Although these two species were originally isolated from tomato, their phylogeny and biological properties are distinct. Despite sharing a high degree of DNA-A nucleotide sequence identity with ToRMV, ToYSV is phylogenetically more closely related to viruses infecting the common weed *Sida*, while ToRMV is closer to other tomato infecting begomoviruses (Andrade et al., 2006). Biologically, the most prominent difference is that ToYSV induces more severe symptoms, with a shorter latent period than ToRMV, in both tomato and the experimental host *Nicotiana benthamiana*. Moreover, ToYSV is efficiently sap-transmissible to several *Nicotiana* species, while ToRMV is poorly sap-transmissible to only a few species, including *N. benthamiana*; neither virus is sap-transmissible to tomato (Calegario et al., 2007; Fernandes et al., 2006). Variations in the length of the period between virus inoculation and the onset of symptoms (defined as the latent period) and in symptom severity suggest distinct levels of virus adaptation to the host, as a consequence of less or more efficient interactions between viral proteins and host factors (Morra and Petty, 2000; Rothenstein et al., 2007). A better adapted virus could presumably replicate at a higher rate, and/or move cell-to-cell or a long distance faster and more efficiently, thus reaching additional tissues besides the phloem, where the virus is initially introduced by the insect vector (Levy and Czosnek, 2003; McGivern et al., 2005; Petty and Qin, 2001; Rojas et al., 2001). Differences in symptom severity could also be a consequence of variations in the suppression of host defense responses by the virus (Fontes et al., 2004; Vanitharani et al., 2004).

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Table 1
Infectivity and latent period in tomato and *N. benthamiana* plants inoculated with ToYSV and ToRMV separately or in combination

	Tomato		*N. benthamiana*	
	Infectivity[a] (% infected plants)	Latent period (dpi)[b]	Infectivity (% infected plants)	Latent period (dpi)
No DNA	0/4 (0)		0/4 (0)	
ToYSV	37/46 (80)	10	27/27 (100)	5
ToRMV	36/49 (74)	14	27/28 (96)	14
ToYSV + ToRMV	42/49 (86)	10	26/26 (100)	5

[a] Number of infected plants/number of inoculated plants, verified by visual observation of symptoms and confirmed by PCR amplification of viral genomic fragments at 28 dpi, using virus-specific primers. Results correspond to the sum of three independent experiments.
[b] Period of time, in days, between inoculation and the onset of systemic symptoms (in non-inoculated leaves).

Here, we report the results of a series of experiments designed to analyze the differences in adaptation of ToYSV and ToRMV to tomato and *N. benthamiana*, and the effects of mixed infection. Patterns of viral DNA accumulation in infected plants, kinetics of the establishment of systemic infection, replication in protoplasts and tissue tropism confirmed the better adaptation of ToYSV to both hosts. Although we found evidence that synergism occurs between the two viruses, both positive and negative interactions were observed in dual infections.

Results

Latent period and symptoms of ToYSV and ToRMV in single or dual infections in tomato and N. benthamiana

Symptoms of ToYSV infection in tomato appeared at 10 dpi (Table 1), and included yellow mosaic, yellow spots and leaf distortion (Fig. 1A), plus a marked reduction in plant growth. In contrast, symptoms of infection by ToRMV appeared at 14 dpi (Table 1), and included mild mosaic and minimal leaf distortion (Fig. 1C), with no evidence of stunting.

The differences between ToYSV and ToRMV infection regarding symptom severity and latent period were even more evident in *N. benthamiana*. Plants inoculated with ToYSV started to display symptoms at 5 dpi, with severe mosaic and leaf curl (Table 1; Fig. 1D). In contrast, plants infected by ToRMV did not display symptoms until 14 dpi, seen as a very mild mosaic and downward cupping of the leaves (Table 1; Fig. 1F).

Tomato plants with dual infection by ToYSV and ToRMV started displaying symptoms at 10 dpi (Table 1; Fig. 1B), similar to plants infected with only ToYSV. Symptoms were more severe than those induced by ToYSV alone, and much more severe than those induced by ToRMV alone. Plants with dual infection displayed a unique leaf roll symptom (Fig. 1B), which was not observed in any of the plants inoculated with either virus alone.

N. benthamiana plants with dual infection by the two viruses started displaying symptoms at 5 dpi, and they were as severe as those observed for single infections by ToYSV (Table 1; Fig. 1E). These results were consistently observed in all three independent experiments.

Kinetics of viral infection in tomato and N. benthamiana

The differences observed between ToYSV and ToRMV in terms of latent period in both hosts suggest that ToYSV reaches the vascular system, and thus initiates a systemic infection, faster than ToRMV. To investigate this hypothesis, we conducted experiments where each virus was inoculated in a single leaf, and this leaf was detached from the plant after different periods of time. The results of these experiments indicate that ToYSV is more efficient than ToRMV at establishing a systemic infection in both hosts (Table 2; Fig. 2).

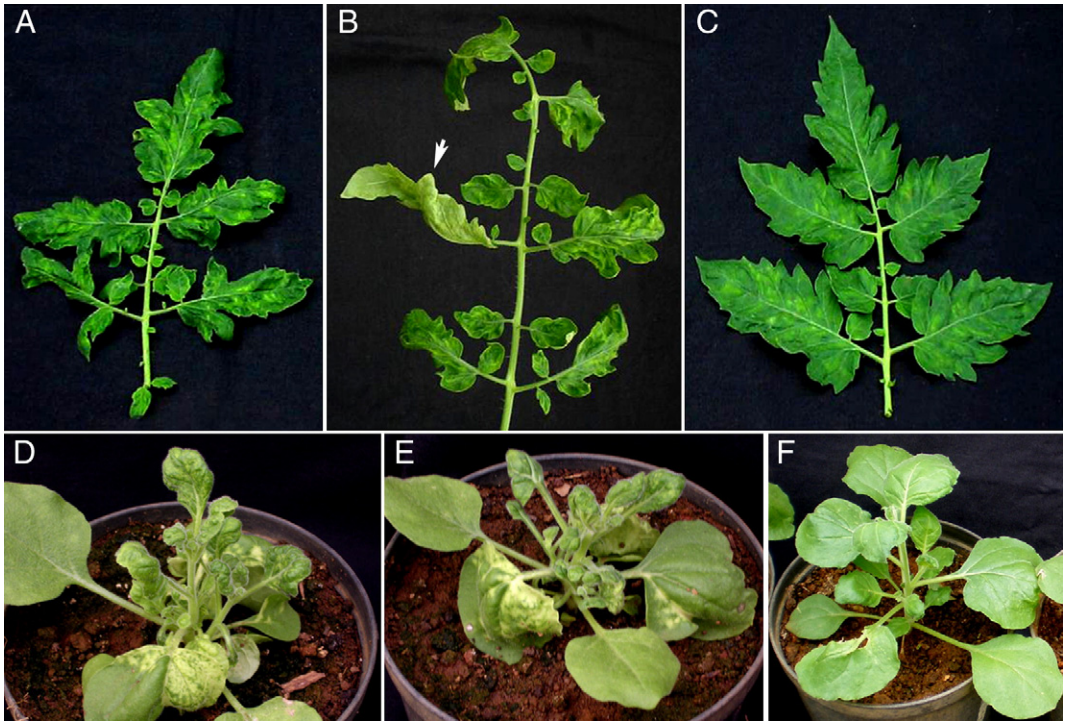

Fig. 1. Symptoms induced by ToYSV and ToRMV in tomato and *Nicotiana benthamiana* plants at 28 days post-inoculation. Tomato plants infected with ToYSV (A) display yellow mosaic and leaf distortion, in contrast to plants infected with ToRMV (C), which display a milder mosaic with minimal leaf distortion. Plants with dual infection (B) display more severe symptoms than those induced by each virus alone. The arrow indicates the leaf roll symptom observed only in plants with dual infection. *N. benthamiana* plants infected with ToYSV (D) display mosaic and severe leaf curl, while those infected with ToRMV (F) display only a mild mosaic and down-cupping of the leaves. Plants with dual infection (E) display symptoms which are similar to those induced by ToYSV alone.

Table 2
Infectivity in tomato and *N. benthamiana* plants inoculated in a single leaf with ToYSV and ToRMV, separately or in combination

Tomato		*N. benthamiana*	
Treatments	Infectivity[a] (% infected plants)	Treatments	Infectivity (% infected plants)
Apex control, ToYSV[b]	4/5 (80)	Apex control, ToYSV	5/5 (100)
Apex control, ToRMV[c]	4/5 (80)	Apex control, ToRMV	5/5 (100)
No DNA	0/10 (0)	No DNA	0/9 (0)
ToRMV, no detachment[d]	2/10 (20)	ToRMV, no detachment	8/10 (80)
ToRMV 4 dpi[e]	1/15 (7)	ToRMV 2 dpi[f]	2/18 (11)
ToRMV 8 dpi	0/16 (0)	ToRMV 4 dpi	1/18 (5)
ToRMV 12 dpi	1/16 (6)	ToRMV 6 dpi	3/16 (19)
ToYSV, no detachment	4/10 (40)	ToYSV, no detachment	11/11 (100)
ToYSV 4 dpi	4/16 (25)	ToYSV 2 dpi	16/26 (62)
ToYSV 8 dpi	1/14 (7)	ToYSV 4 dpi	12/22 (55)
ToYSV 12 dpi	3/15 (20)	ToYSV 6 dpi	18/23 (78)
ToRMV + ToYSV, no detachment	4/12 (30)	ToRMV + ToYSV, no detachment	10/12 (83)
ToRMV + ToYSV 4 dpi[g]	1/15 (7)	ToRMV + ToYSV 2 dpi	3/17 (18)
ToRMV + ToYSV 8 dpi	1/16 (6)	ToRMV + ToYSV 4dpi	9/16 (56)
ToRMV + ToYSV 12 dpi	2/16 (13)	ToRMV + ToYSV 6 dpi	11/16 (69)
ToRMV (ToR + ToY) 4 dpi[h]	0/15 (0)	ToRMV (ToR + ToY) 2 dpi	0/17 (0)
ToRMV (ToR + ToY) 8 dpi	0/16 (0)	ToRMV (ToR + ToY) 4 dpi	1/16 (6)
ToRMV (ToR + ToY) 12dpi	0/16 (0)	ToRMV (ToR + ToY) 6 dpi	0/16 (0)
ToYSV (ToR + ToY) 4 dpi[i]	1/15 (7)	ToYSV (ToR + ToY) 2 dpi	0/17 (0)
ToYSV (ToR + ToY) 8 dpi	0/16 (0)	ToYSV (ToR + ToY) 4 dpi	4/16 (25)
ToYSV (ToR + ToY) 12dpi	1/16 (6)	ToYSV (ToR + ToY) 6 dpi	2/16 (13)

[a] Number of infected plants/number of inoculated plants, verified by visual observation of symptoms and confirmed by PCR amplification of viral genomic fragments at 28 dpi, using virus-specific primers. Results correspond to the sum of three independent experiments.
[b] Inoculation with ToYSV, targeted to the apical meristem.
[c] Inoculation with ToRMV, targeted to the apical meristem.
[d] Inoculated leaf was not detached until 28 dpi.
[e] Tomato plants had the inoculated leaf detached at 4, 8 or 12 dpi.
[f] *N. benthamiana* plants had the inoculated leaf detached at 2, 4 or 6 dpi.
[g] Plants inoculated with both viruses, in which both viruses were present at 28 dpi.
[h] Plants inoculated with both viruses, in which only ToRMV was present at 28 dpi.
[i] Plants inoculated with both viruses, in which only ToYSV was present at 28 dpi.

The latent period in tomato was 10 dpi for ToYSV and 14 dpi for ToRMV (Fig. 2A). In *N. benthamiana*, the latent period was 5 dpi for ToYSV and 14 dpi for ToRMV (Fig. 2C). It is noteworthy that even when a single leaf was inoculated, the latent period and the symptoms induced by both viruses were the same as those observed for plants in which the inoculation was directed at the apical meristem.

Besides having a shorter latent period, ToYSV infected a higher percentage of plants in comparison to ToRMV in single infections at all time periods. In tomato, 25% of the plants in which the inoculated leaf was detached at 4 dpi were infected by ToYSV, and only 7% were infected by ToRMV (Table 2; Fig. 2B). In *N. benthamiana*, 62% of the plants in which the inoculated leaf was detached at 2 dpi were systemically infected by ToYSV, and only 11% by ToRMV (Table 2; Fig. 2D). Equivalent results were observed for the other time periods.

To investigate whether synergism between the two viruses would affect the establishment of a systemic infection, plants were simultaneously inoculated with both viruses in a single leaf. The inoculated leaf was then detached after various times post-inoculation and systemic infection of the plant determined by PCR with virus-specific primers at 28 dpi. In tomato, dual infection caused a significant decrease in the efficiency of systemic infection by ToYSV when the inoculated leaf was detached at 4 dpi (Table 2; Fig. 2B). This negative effect was not observed when the inoculated leaf was detached at 8 or 12 dpi. Systemic infection by ToRMV took place with the same efficiency as that observed for plants inoculated only with this virus when the inoculated leaf was detached at 4 dpi. When the inoculated leaf was detached at 8 or 12 dpi, ToRMV was capable of establishing a systemic infection as efficiently as ToYSV (Table 2; Fig. 2B). These results suggest that ToRMV has a negative effect on ToYSV during the initial stages of infection, but ToYSV facilitates systemic infection by ToRMV at the later stages of infection.

The negative interference of ToRMV on ToYSV during the initial stages of infection was more evident in *N. benthamiana*, a host in which both viruses were more efficient in the establishment of a systemic infection compared to tomato. In plants where the inoculated leaf was detached at 2 dpi, ToYSV systemically infected 62% of the plants in single infection, and only 18% of the plants in dual infection (Table 2; Fig. 2D). The percentage of plants infected by ToRMV was similar in single and dual infection (11 and 18%, respectively, with no statistical difference). However, when the inoculated leaf was detached at 4 or 6 dpi, not only was there no negative effect on ToYSV, but the percentage of plants infected by ToRMV increased drastically compared to plants with a single infection by this virus (Table 2; Fig. 2D).

Together, these results indicate that ToYSV is better adapted than ToRMV to both hosts. ToYSV is capable of establishing a systemic infection faster and more efficiently than ToRMV in both hosts, although both viruses are more efficient in *N. benthamiana* than in tomato. More interestingly, ToRMV has a negative effect over ToYSV during the initial stages of dual infection, but ToYSV eventually facilitates systemic infection by ToRMV at the later stages, in both hosts.

Accumulation of ToYSV and ToRMV in single or dual infections in tomato and N. benthamiana

In order to determine whether symptom severity could be correlated with viral DNA accumulation in infected tissues, systemically infected leaves were collected at 14 and 28 dpi, total DNA was extracted and used as a template for quantitative, real-time PCR (qPCR) with virus-specific primers.

In tomato, the results of three independent experiments indicate that, for plants infected with a single virus, the accumulation of ToYSV DNA was greater than that of ToRMV at 14 dpi, but the opposite was observed at 28 dpi (Fig. 3). Furthermore, the amount of ToYSV DNA decreased between the two time points, while that of ToRMV increased significantly from 14 to 28 dpi (Fig. 3).

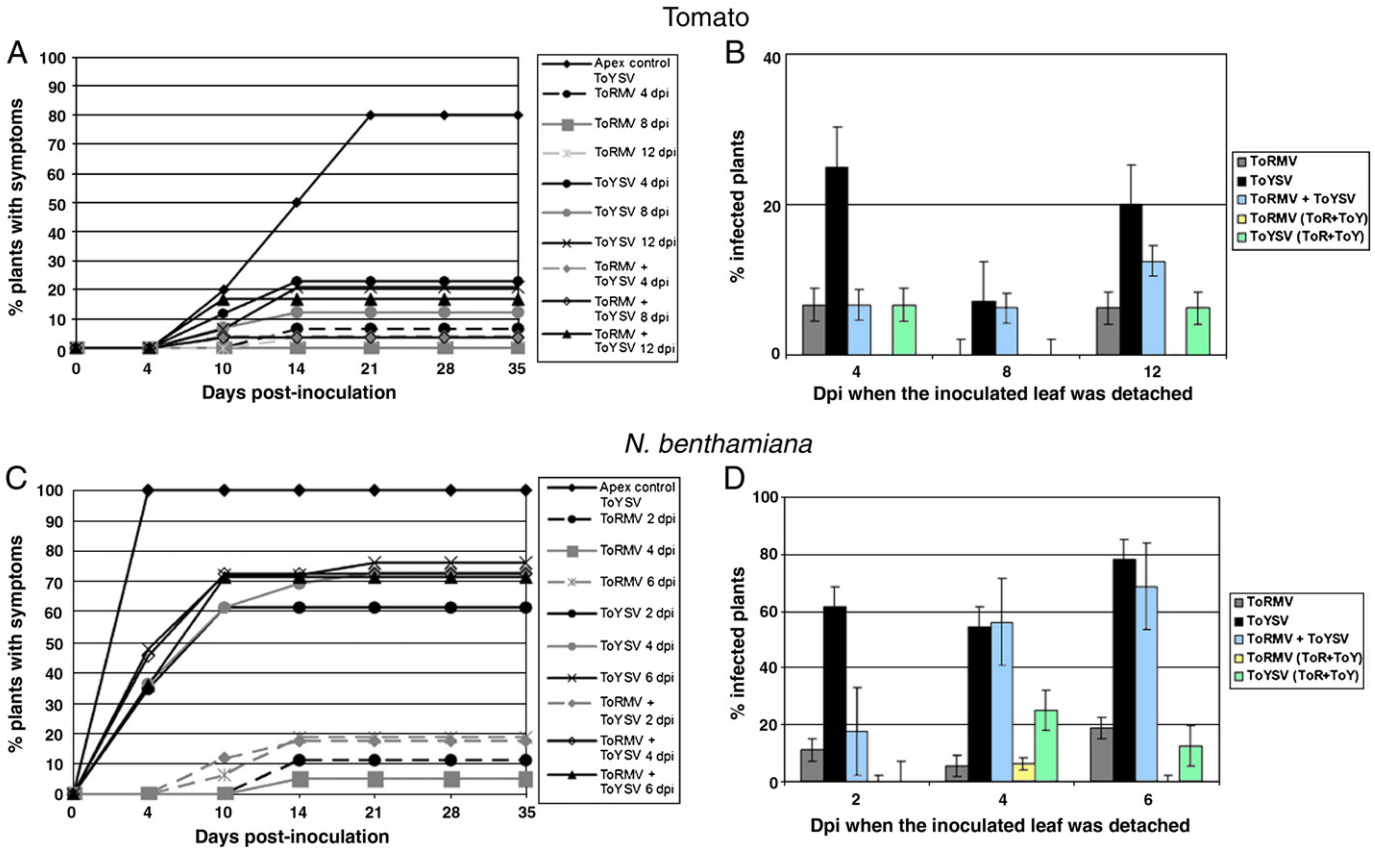

Fig. 2. Kinetics of the establishment of systemic infection in tomato and *N. benthamiana* plants infected with ToYSV and ToRMV, alone or in combination, when the inoculated leaf was detached from the plant at 4, 8 and 12 days post-inoculation (dpi) (tomato) or 2, 4 and 6 dpi (*N. benthamiana*). (A) and (C), Percentage of tomato and *N. benthamiana* plants, respectively, displaying systemic symptoms at different time points following inoculation, for each treatment. (B) and (D), Percentage of tomato and *N. benthamiana* plants, respectively, in which viral infection was confirmed by PCR at 28 dpi using species-specific primers, for each treatment. Treatments as in Table 2.

In dual infection of tomato plants, significant decreases were observed in viral DNA accumulation for both viruses (Fig. 3). Furthermore, both viruses had statistically significant decreases from 14 to 28 dpi (Fig. 3). These results, indicating negative interference, were consistently observed in three independent experiments.

In single infections in *N. benthamiana*, ToYSV reached a significantly higher titer than ToRMV at both time points, and both viruses decreased from 14 to 28 dpi (Fig. 3). As observed in tomato, a decrease in DNA accumulation was observed for both viruses in dual infection of this host, except that the accumulation of ToRMV increased significantly from 14 to 28 dpi (Fig. 3). In fact, ToRMV reached a higher titer in the dual infection compared to single infection at 28 dpi.

Together, these results indicate negative interference between the two viruses in tomato, while both negative interference and synergism take place in dual infection in *N. benthamiana*. In this host, synergism is correlated with an increased accumulation of ToRMV (at 28 dpi) but not ToYSV. Furthermore, in single infection, ToYSV accumulates to a higher level than ToRMV in this host, suggesting that ToYSV is better adapted and is probably the virus providing the factor(s) responsible for the synergistic interaction.

Replication of ToYSV and ToRMV in N. benthamiana protoplasts

To investigate possible differences in the viral replication rate, infectious clones of the DNA-A of ToYSV and ToRMV were electroporated separately or in combination into *N. benthamiana* protoplasts. Cells were collected at 48 and 96 h post-electroporation (hpe), total DNA was extracted and used as a template for qPCR with virus-specific primers. The results indicate that ToYSV reaches a higher titer than ToRMV in single and dual infections, at both time points (Fig. 4). However, the accumulation of both viruses is decreased from 48 to 96 hpe (Fig. 4). Interestingly, no increase in replication was observed in dual infection compared to single infections, for either virus (Fig. 4). Actually, compared to single infections, the concentration of ToYSV remained constant until 48 hpe but was reduced at 96 hpe, while the opposite was observed for ToRMV (Fig. 4).

These results could explain, at least in part, the striking differences in symptom severity by the two viruses in this host, as well as the differences in the establishment of a systemic infection, and support the conclusion that ToYSV is a better adapted virus. However, they do not explain the increase in ToRMV titer observed in the dual infection when leaf tissues were analysed (Fig. 3), indicating that synergism in *N. benthamiana* is not associated with viral replication.

Tissue tropism of ToYSV and ToRMV in tomato and N. benthamiana

To test the hypothesis that differential tissue tropism is responsible for the differences in symptom severity between ToYSV and ToRMV and for the higher DNA accumulation of ToYSV compared to ToRMV, *in situ* hybridization studies were carried out in systemically infected leaves of tomato and *N. benthamiana* plants with single or dual infection.

In tomato, both ToYSV and ToRMV were detected only in cells associated with the vascular tissues (Table 3; Supplementary Fig. S1). In *N. benthamiana*, ToYSV was detected in a large number of mesophyll cells, in addition to vascular tissues (Table 3; Fig. 5). In contrast, ToRMV remained phloem-restricted in this host. It was remarkable that both viruses infected the same proportion of vascular cells in both

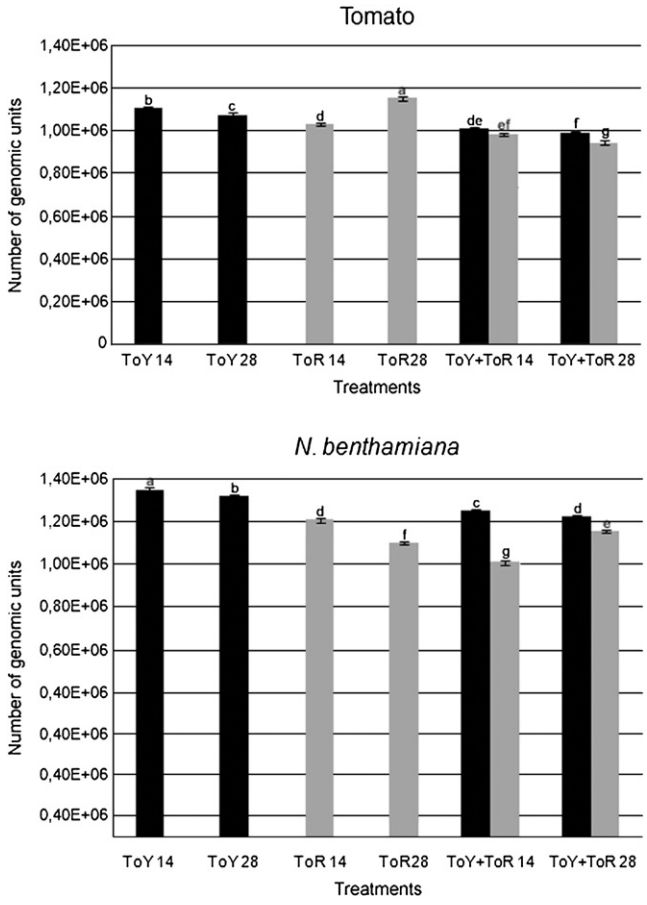

Fig. 3. Viral DNA accumulation in tomato and *Nicotiana benthamiana* plants inoculated with ToYSV and ToRMV separately or in combination. Total DNA was extracted from systemically infected leaves at 14 and 28 days post-inoculation and used as a template for quantitative, real-time PCR (qPCR) with virus-specific primers. Bars with the same letter correspond to DNA amounts which do not differ statistically according to Tukey's test ($p<0.01$).

hosts, indicating that this factor by itself does not explain the differences in infection kinetics and DNA accumulation. When semithin sections from plants infected with both viruses were analyzed using the ToRMV-specific probe, viral DNA was detected in mesophyll cells (Table 3; Fig. 5), indicating that dual infection with ToYSV allowed mesophyll invasion by ToRMV. Again, both viruses

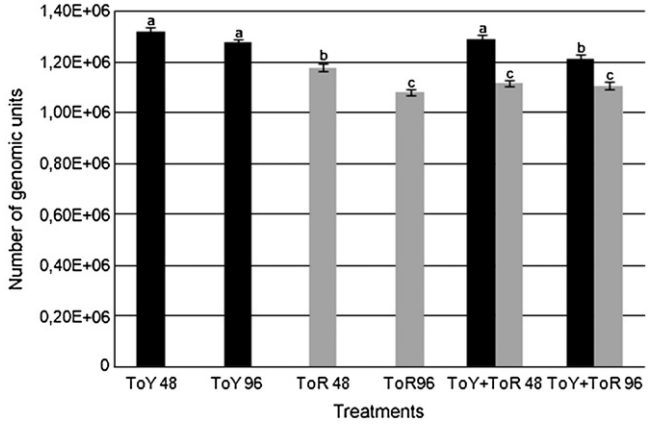

Fig. 4. Replication of ToYSV and ToRMV in *Nicotiana benthamiana* protoplasts. Protoplasts were electroporated with infectious clones of the DNA-A of ToYSV and ToRMV, separately or in combination. Cells were collected at 48 and 96 h post-electroporation, total DNA was extracted and used as a template for quantitative, real-time PCR (qPCR) with virus-specific primers. Bars with the same letter correspond to DNA amounts which do not differ statistically according to Tukey's test ($p<0.01$).

Table 3
Tissue tropism of ToRMV and ToYSV in tomato and *Nicotiana benthamiana*, in single or dual infections

Treatments	Tomato		*N. benthamiana*	
	Mesophyll	Vasculature	Mesophyll	Vasculature
ToRMV (ToRMV probe)	28/1773 (2)[a]	164/497 (33)	45/1903 (2)	165/492 (34)
ToYSV (ToYSV probe)	39/1810 (2)	153/458 (33)	356/1776 (20)	181/545 (33)
ToRMV + ToYSV (ToRMV probe)	3/1844 (0.1)	143/435 (33)	333/1675 (20)	137/412 (33)
ToRMV + ToYSV (ToYSV probe)	18/1582 (1)	142/428 (33)	305/1523 (20)	144/425 (34)
ToRMV (ToYSV probe)	0/1745 (0)	6/470 (1)	0/1935 (0)	4/512 (0.7)
ToYSV (ToRMV probe)	2/1899 (0.1)	1/463 (0.2)	3/1732 (0.1)	2/430 (0.4)
Mock (ToRMV probe)	0/1792 (0)	0/427 (0)	0/1814 (0)	0/462 (0)
Mock (ToYSV probe)	0/1786 (0)	0/500 (0)	0/1821 (0)	0/468 (0)

Nuclei from infected mesophyll or vascular cells were counted in non-consecutive semi thin sections prepared from infected plants and hybridized with fluorescently-labeled virus-specific probes. Total number of nuclei were counted based on DAPI staining.
[a] Number of infected nuclei/total number of nuclei counted (percentage of infected nuclei).

infected the same proportion of mesophyll cells, although our assay could not determine whether the same cells were infected by both viruses. The ToRMV-specific probe did not detect ToYSV in plants with a single infection by this virus, thus confirming its specificity (Table 3).

Together, these results indicate that the differences in symptom severity and DNA accumulation observed between ToYSV and ToRMV in tomato are not due to differences in tissue tropism, since both viruses are phloem-restricted in this host. However, tissue tropism could, at least in part, explain the differences observed in *N. benthamiana*, since ToYSV is capable of invading the mesophyll in this host, unlike ToRMV. Furthermore, in dual infections, the fact the ToRMV is no longer confined to the phloem could explain its increased DNA accumulation at the late stages of infection.

Discussion

In Brazil, at least eight begomoviruses are currently a major threat to tomato production (Fernandes et al., 2008; Ribeiro et al., 2003). The introduction of a new biotype (biotype B) of the insect vector *Bemisia tabaci*, which unlike the previously present biotype (biotype A) efficiently colonizes solanaceous plants such as the tomato, allowed the transfer of indigenous viruses infecting wild and/or weed hosts to tomato. A few of these species have became prevalent in the field (Ambrozevicius et al., 2002; Castillo-Urquiza et al., 2008; Fernandes et al., 2008; Ribeiro et al., 2003), indicating adaptation to the newly available host.

Two of the begomovirus species infecting tomato in the state of Minas Gerais, *Tomato yellow spot virus* (ToYSV) and *Tomato rugose mosaic virus* (ToRMV), exemplify the phenomenon described above. Symptoms of ToYSV infection in tomato appear at 10 dpi and are considerably more severe than those induced by ToRMV, which appear at 14 dpi. These differences are even more evident in the experimental host *N. benthamiana*, and in potential wild hosts such as *Nicotiana glutinosa* and *Nicotiana tabacum* (data not shown). Dual infection with both viruses caused symptoms to appear at 10 dpi in tomato and 5 dpi in *N. benthamiana*. In tomato, symptoms were more severe than those induced by each virus alone. In *N. benthamiana*, symptoms were comparable to those induced by ToYSV alone.

The efficiency of a virus in establishing a systemic infection, as expressed by latent period and the percentage of plants infected following inoculation, may reflect a better interaction between viral proteins and host factors, leading to improved replication, cell-to-cell

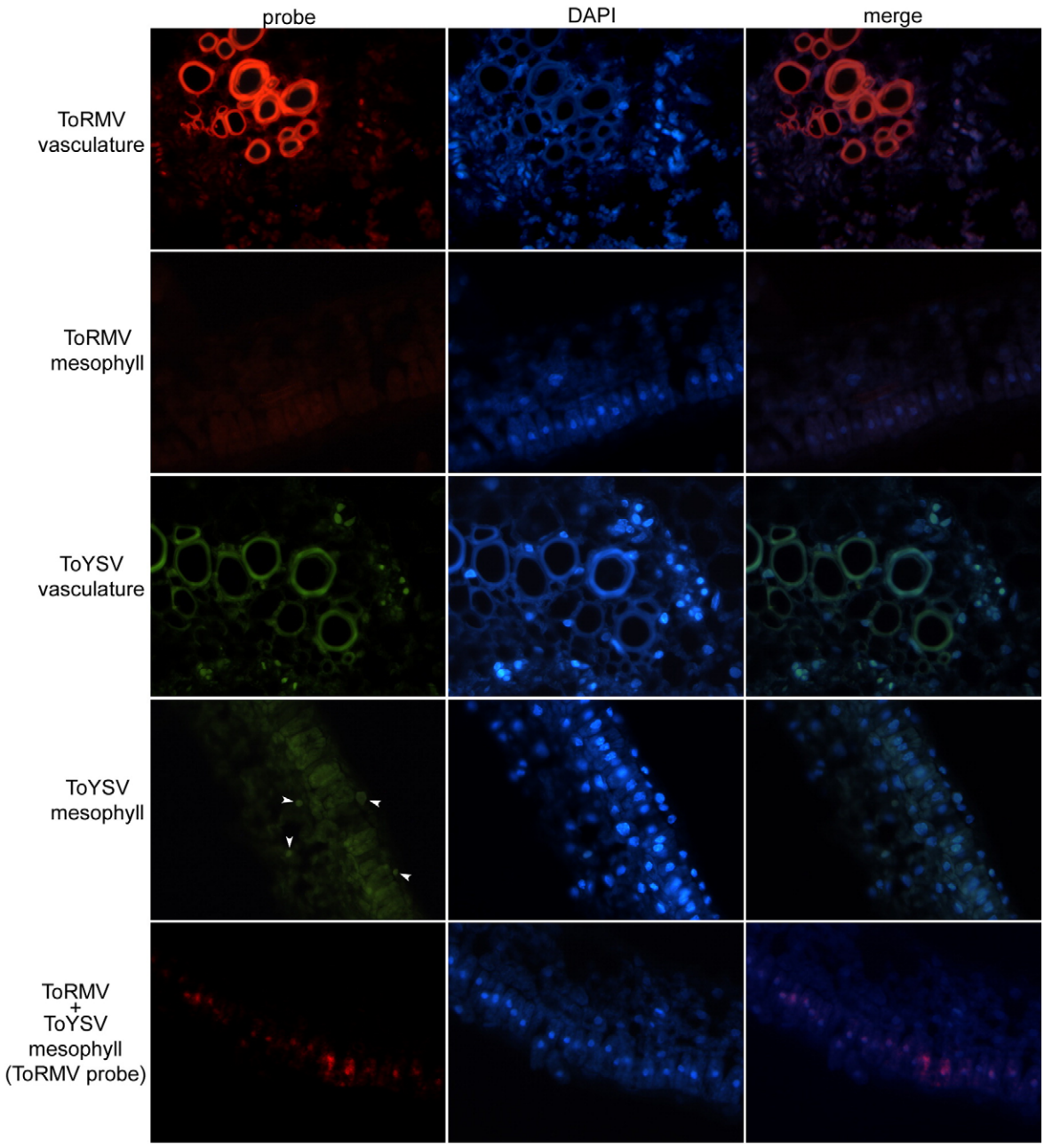

Fig. 5. Localization of ToYSV and ToRMV in *N. benthamiana* by *in situ* hybridization at 28 days post-inoculation. Semi thin sections prepared from single- and dual-infected plants were hybridized with virus-specific probes and examined by fluorescence microscopy. ToRMV is phloem-restricted (compare "ToRMV vasculature" and "ToRMV mesophyll"), while ToYSV infects both the vasculature and the mesophyll. In dual infection, ToRMV is released from the phloem and invades the mesophyll (ToRMV + ToYSV, mesophyll). The strong background in "ToYSV vasculature" is due to chloroplast autofluorescence in the fluorescein channel; arrowheads indicate infected nuclei.

movement and/or suppression of host defense responses. Likewise, reduced levels of viral DNA accumulation in hosts to which the virus is poorly adapted probably reflect an inefficient interaction between viral and host factors (Petty et al., 1995).

ToYSV is more efficient than ToRMV in establishing a systemic infection in both tomato and *N. benthamiana*, and both viruses are more efficient in infecting the experimental host *N. benthamiana* compared to tomato. It must be considered that both hosts belong to the Solanaceae, that both viruses infect a number of solanaceous species, and that symptoms are particularly severe in *Nicotiana* species such as *N. glutinosa* and *N. rustica*, besides *N. benthamiana* (Calegario et al., 2007; Fernandes et al., 2006). Therefore, it is perfectly possible that *Nicotiana* species are the natural host of these viruses (or of the viruses from which they evolved). It is noteworthy that in an initial experiment in which the inoculated leaf was detached from tomato plants at the same periods of time used for *N. benthamiana* (2, 4 and 6 dpi), the percentage of infected plants was close to zero (data not shown). This further highlights the lower efficiency of both viruses in infecting tomato compared to *N. benthamiana*.

Differences in host adaptation of begomoviruses were demonstrated by Hou et al. (1998), working with *Bean dwarf mosaic virus* (BDMV) and *Tomato mottle virus* (ToMoV). BDMV was more pathogenic than ToMoV in the experimental hosts *N. benthamiana* and *N. tabacum* (susceptible to both viruses), and both viruses were less efficient in infecting their respective "natural" hosts (common bean and tomato).

Intriguing results were observed in plants inoculated with both viruses in this study. When the inoculated leaf was detached at 2 dpi

(in *N. benthamiana*) or 4 dpi (in tomato), a significant reduction was observed in the percentage of plants infected by ToYSV in comparison to plants inoculated with only this virus. This negative interference of ToRMV with ToYSV was not observed at the other time points (4 and 6 dpi for *N. benthamiana*, 8 and 12 dpi for tomato). On the contrary, in these treatments the percentage of infected plants by ToYSV was equivalent to the one observed in single infection, while the percentage of plants infected by ToRMV increased. These results suggest that the presence of ToRMV interferes with initial events of the ToYSV infection cycle, which could be related to replication in the initially infected cell or cell-to-cell movement. This hypothesis is further supported by the results of viral DNA accumulation, which indicate that both viruses reach lower titers in dual infection compared to single infections, in both hosts (with the single exception of ToRMV in *N. benthamiana* at 28 dpi). It must be considered that simultaneous inoculation with both viruses does not necessarily mean that both viruses will be present in the same cells, although it is reasonable to assume that at least some cells will have both viruses, as reported for mixed infection with *Tomato yellow leaf curl virus* (TYLCV) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) in tomato and *N. benthamiana*, in which 20% of the cells were infected by both viruses (Morilla et al., 2004).

In a dual infection, viral proteins which are less efficient in interacting with host factors could have a negative effect in the interaction of the more efficient proteins, a phenomenon known as negative dominance (Herskowitz, 1987). An example of negative dominance is the inhibition of ACMV infection by co-inoculation of a mutant virus in which the coat protein gene was replaced by a truncated version of the MP gene of *Tomato golden mosaic virus* (TGMV) (von Arnim and Stanley, 1992). The use of negative dominance has been the basis for a number of attempts to generate geminivirus resistant transgenic plants (Antignus et al., 2004; Duan et al., 1997; Hou et al., 2000; Noris et al., 1996; Shivaprasad et al., 2006). In any event, whichever is the nature of this negative interference, once the infection cycle progresses to intermediate and late events, not only does ToYSV infect the plant more efficiently, but it actually assists ToRMV in the establishment of a systemic infection. In *N. benthamiana*, this could be explained by ToYSV allowing ToRMV to invade the mesophyll, suggesting a movement-related interaction. In tomato, our data failed to indicate the nature of the positive interaction between the two viruses, although differences in replication rate and tissue tropism can be ruled out.

Both synergism and interference between geminiviruses and viruses from different genera and families have been described. Synergism is well documented among cassava-infecting geminiviruses as a consequence of improved RNA silencing suppression (Fondong et al., 2000; Pita et al., 2001; Vanitharani et al., 2004). Negative interference was observed in dual infections between *Abutilon mosaic virus* (AbMV), a begomovirus, and the tobamoviruses *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV), with a reduction in DNA accumulation and infectivity of AbMV in *N. benthamiana* (Pohl and Wege, 2007). However, we believe this to be the first time that both positive (synergism) and negative (interference) interactions are verified for the same interaction at different stages of infection.

In single infections, the differences observed in the severity of symptoms correlate with viral DNA accumulation in both hosts: ToYSV accumulates to a higher level compared to ToRMV at both 14 and 28 dpi. An equivalent result was observed in cassava and *N. benthamiana* plants infected with *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) from Uganda (Pita et al., 2001). In our case, the method used to estimate viral DNA accumulation does not allow us to conclude whether the differences observed are due to higher replication or to a greater number of infected cells (for example, due to differences in tissue tropism).

In dual infections, the accumulation of both viruses decreased in tomato, and ToYSV decreased in *N. benthamiana* as well. However, the accumulation of ToRMV was higher than in single infection in *N. benthamiana*. These results indicate that synergism does take place between the two viruses in this host. It must be pointed out that, for geminiviruses at least, synergism, as expressed by increased symptom severity in dual infection compared to single infections, does not necessarily lead to increased DNA accumulation, as demonstrated in tomato and *N. benthamiana* plants infected with TYLCV and TYLCSV (Morilla et al., 2004).

Viral replication assays in *N. benthamiana* protoplasts indicated that the replication rate of ToYSV is higher than that of ToRMV in this host. These results suggest that the ToYSV replication-associated proteins interact better with host factors. Together with the results from the infection kinetics experiment, this indicates that ToYSV accumulates DNA (and consequently synthesizes mRNA and protein) faster than ToRMV, and may therefore be capable of establishing a systemic infection more efficiently, possibly by evading host defense responses. In dual infections, although the replication rate of ToYSV is higher than ToRMV at both time points, ToYSV accumulation is significantly reduced at 96 hpe, reinforcing the observation that ToRMV negatively interferes with ToYSV replication.

Infection by a number of geminiviruses is restricted to cells of the vascular tissues (Hoefert, 1987; Horns and Jeske, 1991; Morilla et al., 2004; Rojas et al., 2001; Wang et al., 1996). However, many begomoviruses are capable of infecting mesophyll cells (Morra and Petty, 2000; Rushing et al., 1987; Sudarshana et al., 1998; Wang et al., 1996; Wege et al., 2000). This capacity to invade tissues in addition to the vasculature indicates a better adaptation of the virus to its host, and is normally associated with greater severity of symptoms and with sap-transmissibility (Morra and Petty, 2000; Petty and Qin, 2001; Wege et al., 2000; reviewed by Rojas et al., 2005).

In situ hybridization of leaf sections from tomato plants with single or dual infections detected the presence of viral DNA only in cells of the vasculature. A similar result was observed for tomato infection by TYLCV and TYLCSV, both of which were phloem-restricted either in single or dual infections (Morilla et al., 2004). Therefore, tissue tropism does not explain the differences between ToYSV and ToRMV in single infection or the more severe symptoms observed in dual infection. We did not carry out protoplast replication assays in tomato, and therefore this remains as a possible explanation for these differences. Another plausible hypothesis would be that ToYSV encodes a more efficient silencing suppressor than ToRMV. As mentioned above, synergism between cassava-infecting geminiviruses was associated with enhanced silencing suppression (Vanitharani et al., 2004).

In *N. benthamiana*, *in situ* hybridization detected ToYSV in both phloem-associated and mesophyll cells, while ToRMV remained phloem-restricted in single infection. Therefore, not only does ToYSV replicates at a higher rate than ToRMV, but the fact that ToYSV is capable of invading the mesophyll could explain its higher DNA accumulation and more severe symptoms.

In situ hybridization from foliar sections of *N. benthamiana* plants with dual infection indicated the presence of ToRMV in mesophyll cells. This demonstrates that the presence of ToYSV allows ToRMV to invade the mesophyll, and could explain the higher accumulation of this virus even though its replication rate was unchanged compared to the single infection. Such an effect is not unusual for geminiviruses. Dual infection by TGMV, which invades mesophyll cells of *N. benthamiana*, and *Bean golden mosaic virus* (BGMV) or ACMV, both phloem-restricted in this host, resulted in BGMV and ACMV invading the mesophyll (Morra and Petty, 2000; Wege et al., 2001). Release of phloem restriction has been observed even between geminiviruses and viruses from distinct genera, such as TMV (Carr and Kim, 1983) and *Cucumber mosaic virus* (CMV) (Wege and Siegmund, 2007), indicating that the viral factors involved in movement are less specific than those involved in replication.

In summary, results from experiments determining kinetics of systemic infection, viral DNA accumulation, replication in protoplasts

and tissue tropism indicate that ToYSV is more efficient in carrying out the early (pre-systemic) events of the viral infection cycle, reaching a higher concentration and establishing a systemic infection sooner than ToRMV in both tomato and *N. benthamiana*. ToRMV negatively interferes on ToYSV during these initial stages of infection, but once a systemic infection is established, this negative interference ceases. In *N. benthamiana*, ToYSV invades the mesophyll, while ToRMV is phloem-restricted. During dual infection in this host, ToYSV releases ToRMV from the phloem. Based on these results, we conclude that ToYSV is better adapted than ToRMV, although its better adaptation is more evident in *N. benthamiana* than in tomato. In tomato, the better adaptation of ToYSV is expressed by its capacity of reaching a higher concentration in a shorter period of time, which may lead to a higher concentration of viral virulence factors such as proteins involved in the suppression of host defense responses early in the infection. In *N. benthamiana*, the better adaptation of ToYSV is further expressed by its ability to invade mesophyll cells. Our future studies will focus on the identification and functional analysis of the viral virulence factors responsible for the better adaptation of ToYSV compared to ToRMV.

Material and methods

Viral isolates and plant material

Infectious clones of the virus isolates ToYSV-[Bic2] (Andrade et al., 2006) and ToRMV-[Ube1] (Fernandes et al., 2006) were used in all experiments. Tomato (*Solanum lycopersicum* cv. "Santa Clara") and *Nicotiana benthamiana* plants were biolistically inoculated (Aragão et al., 1996) using 2 μg of each genomic component (DNA-A and DNA-B). Inoculated plants were kept in a greenhouse with average daily temperatures of 26 ± 2 °C.

Kinetics of viral infection

Tomato and *N. benthamiana* plants were inoculated with ToYSV only, ToRMV only, and ToYSV plus ToRMV, but with the inoculation targeting a single leaf, which was detached from the plant at 2, 4 and 6 days post-inoculation (dpi) for *N. benthamiana* and 4, 8 and 12 dpi for tomato. Inoculated plants were evaluated for symptom expression for 35 days. DNA from all plants was extracted at 28 dpi as described (Dellaporta et al., 1983) and used as a template for PCR-amplification of viral genomic fragments using virus-specific primers (ToYSV: 5′GCT GAG GCG TTA AAT GCT CC3′ and 5′ATG TCA GGA ATG CCT GGT GG3′; ToRMV: 5′GGT AGG ATC CTG GTA TTT TCC AGC3′ and 5′GGG GGA ATT CAT GAT GCA TTT GAC GAG G3′) to confirm the presence of each virus. Three independent experiments were performed.

Viral DNA accumulation in infected plants

Tomato and *N. benthamiana* plants were inoculated in the apical meristem with the same combinations of the previous experiment. Approximately 0.3 g of symptomatic leaves were collected at 14 and 28 dpi, ground in liquid nitrogen, transferred to a microfuge tube containing 1 ml of extraction buffer (100 mM Tris–HCl pH 8.0, 200 mM NaCl, 2 mM EDTA, 1% β-mercaptoethanol) and 50 μl of 20% SDS, and incubated at 65 °C for 10 min. After phenol:chloroform extraction, the DNA was precipitated with 0.7 vols of isopropanol, washed with 70% ethanol and ressuspended in 200 μl of TE (10 mM Tris–HCl pH 7.5, 1 mM EDTA).

Viral DNA accumulation was determined by quantitative real-time PCR (qPCR). Reactions were prepared in a final volume of 25 μl, using the Plexor qPCR System (Promega) and an ABI7500 thermal cycler (Applied Biosystems), following manufacturer's instructions. The PCR protocol included an initial denaturing step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 35 s, followed by a dissociation stage. Virus-specific primers (Supplementary Table S1) were designed using the Plexor™ Primer Design Software (Promega), and their specificity was tested in qPCR reactions using plasmid DNA containing the complete DNA-A of each virus (10^6 copies per reaction). For viral DNA quantification, standard curves were prepared using serial dilutions of plasmid DNA containing the complete DNA-A of either ToRMV or ToYSV (10^0 to 10^6 copies of the viral genome per reaction). Standard curves were obtained by regression analysis of cycle threshold (Ct) values of each one of the three replications of a given dilution in relation to the log of the amount of DNA in each dilution. For absolute quantification of the number of viral DNA molecules in the different treatments, 50 ng of total DNA, extracted as described previously, were used in multiplex reactions containing both sets of virus-specific primers. Each sample was analyzed in triplicates, and three biological replications of the experiment were carried out. Analyses were carried out using the Plexor™ Analysis software version 1.1.4 (Promega). The data was subjected to statistical analysis and the means were compared using Tukey's test ($p<0.01$).

Viral replication in protoplasts

A *N. benthamiana* cell suspension culture was maintained according to Hall (1991), by incubation at 26 °C and 90 rpm and weekly subculturing at a dilution of 1:10. Protoplasts were isolated from the cell suspension culture according to Qi and Ding (2002), with some modifications. Cells were collected by centrifugation at 70 g for 5 min , resuspended in solution I (0.5 M mannitol, 3.6 mM MES, pH 5.5) containing 1.5% cellulase "Onozuka" R-10 (Yakult Honsha), 0.4% macerozyme R-10 (Yakult Honsha) and 0.2% driselase (Sigma), and incubated in the dark at room temperature for approximately 4 h at 40 rpm. The suspension was passed through a 64 mesh sieve (Wilson Sieves) and centrifuged at 50 g for 10 min. Protoplasts were washed twice with solution I and ressuspended in 200 μM MOPS pH 7.2, 5 mM KCl, 0,5 M mannitol. The concentration was adjusted to 5×10^6 cells/ml. Protoplasts were electroporated in 0.4 cm cuvettes at 250 V and 500 μF, with 20 μg of each genomic component and 30 μg of salmon sperm DNA. After electroporation the suspension was kept on ice for 10 min, diluted in 10 ml of MSP1 medium (MS salts supplemented with 0.5 mg/l of 6-benzylaminopurine, 2 mg/l of α-naphtaleneacetic acid, 3% sucrose and 0.5 M mannitol pH 5.8) and incubated at 26 °C in the dark. Protoplasts were collected at 48 and 96 h post-electroporation (hpe) and total DNA was extracted as described (Hou et al., 1998). Viral DNA accumulation was analyzed by qPCR, as described above. Three independent experiments were performed.

In situ hybridization

Leaves in the second internode of tomato and *N. benthamiana* plants inoculated with the same combinations of the previous experiments were collected 28 dpi, fixed in 4% paraformaldehyde for 16 h , dehydrated in ethanol (10–100%) and paraffin embedded. Sections of 10 μm were prepared and fixed in slides treated with (3-aminopropyl)tirethoxy-silane. Paraffin wax was removed with xylol. The sections were treated with proteinase K (1 μg/ml) for 15 min at 37 °C, and washed with water. The ToYSV probe corresponded to nucleotides 1423-2148 of the DNA-A, obtained after Pst I digestion of clone pToYSV-A 1.2 (Andrade et al., 2006). The ToRMV probe corresponded to nucleotides 1711-2147 of the DNA-A, obtained after Cla I and EcoR I digestion of clone pUb1-49 (Fernandes et al., 2006). Probes were labeled by random priming with tetramethyl-rhodamine-5-dUTP (ToRMV) or fluorescein-12-dUTP (ToYSV) (Roche Applied Sciences), according to the manufacturer's instructions.

Sections were hybridized for 18 h in hybridization solution (22 mM Tris–HCl pH 7.5, 50% formamide, 0.6 M NaCl, 144 μl 50×

Denhardt's solution, 0.12% dextran sulfate, 40 mM EDTA, 500 μg/ml of salmon sperm DNA, and 600 ng/ml of probe) at 42 °C in a moist chamber. After hybridization, sections were washed sequentially in 1× SSC at room temperature, twice in 1× SSC at 55 °C for 15 min, twice in 0.5× SSC at 55 °C for 15 min and once in 0.5× SSC for 10 min at room temperature. Sections were stained with 4′,6-diamidino-2-phenylindole (DAPI), mounted in water and photographed using an Olympus BX-61 microscope with an attached Q-Color3 digital camera (Olympus Optical). Images were processed using Adobe Photoshop 7.0. Overlays were produced by setting the opacity of the rhodamine and fluorescein layers to 70%.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.01.046.

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