Synergistic pathogenicity of a phloem-limited begomovirus and tobamoviruses, despite negative interference

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In contrast to previous observations on phloem-limited geminiviruses supported in movement and accumulation by RNA viruses such as cucumo- and tobamoviruses, tissue infiltration by Abutilon mosaic virus (AbMV) was enhanced by neither Tobacco mosaic virus (ToMV) in two different hosts, Nicotiana benthamiana and tomato. Both tobamoviruses exerted a negative effect on the DNA virus, resulting in a decrease in AbMV accumulation and significantly reduced infectivity in N. benthamiana. Despite these unexpected molecular observations, a striking synergistic enhancement in pathogenicity occurred with respect to stunting and necrosis. In situ hybridization revealed that this was not due to any alteration of tissue infiltration by AbMV, which also remained limited to the phloem in the mixed infections. Transgenically expressed ToMV 30K movement protein was not able to induce phloem escape of AbMV in tomato plants and did not lead to any obvious change in begomovirus symptomatology.

Significant proportions of crop and ornamental plants from all over the world have been shown to be infected by two or more distinct viruses (Lesemann & Winter, 2002). This contrasts with relatively scarce analyses of the molecular and ecological consequences of mixed infections with unrelated viruses (reviewed by Hull, 2002). Recent experiments have revealed that symptom induction, accumulation and movement by the single-stranded (ss) DNA-containing begomovirus Abutilon mosaic virus (AbMV) in the economically important family Geminiviridae (Stanley et al., 2005) are enhanced strongly by the positive-sense (+) ssRNA virus Cucumber mosaic virus (CMV) (Wege & Siegmund, 2007). Tobacco mosaic virus (TMV) and related (+)ssRNA tobamoviruses (Lewandowski, 2005) comprise one of the few further genera analysed in more detail with regard to interactions with simultaneously inoculated viruses. Most of the co-infection and complementation assays have focused on entire or partial genomes of other (+)ssRNA viruses (Atabekov et al., 1999; Bendahmane et al., 1995; Cooper et al., 1996; De Jong & Ahlquist, 1992; Hacker & Fowler, 2000; Hamilton & Nichols, 1977; Malyschenko et al., 1989; Martin et al., 2004; Ryabov et al., 1999; Solovyev et al., 1996; Taliantsky et al., 1992). The experiments revealed a range of observations, from unilateral suppression of virus accumulation (Bendahmane et al., 1995) via spread recovery (Malyschenko et al., 1989) to enhancement, some of them including symptom synergism. Only a few analyses have addressed the interplay of tobamoviruses with DNA viruses. Two reports have focused on interactions with geminiviruses (Carr & Kim, 1983; Sunter et al., 2001), which cause devastating epidemics in crops such as cassava and legumes and in members of the Solanaceae (Legg & Fauquet, 2004; Mansoor et al., 2003; Rybicki & Pietersen, 1999). These two publications presented data in which at least one of the viruses was supported by another unrelated virus: Carr & Kim (1983) showed by electron microscopy that co-infection with a tobamovirus leads to release of the otherwise phloem-limited begomovirus Bean golden mosaic virus (BGMV) into mesophyll parenchyma of beans, and Sunter et al. (2001) showed that transgenically delivered (A)C2 proteins of the begomovirus Tomato golden mosaic virus (TGMV) or the curtovirus Beet curly top virus (BCTV) increased the rate of TMV infection in Nicotiana species (‘enhanced-susceptibility’ phenotype).

On the basis of these prior examples, we wanted to determine whether positive tobamovirus–geminivirus interactions were typical of the interactions between these two genera, examining co-infections of TMV or Tomato mosaic virus (ToMV) with the phloem-limited AbMV (Horns & Jeske, 1991; Jeske, 2000; Wege et al., 2000, 2001). We aimed to correlate effects on the symptoms’ phenotype...
with molecular observations on virus accumulation and with viral tissue tropism at the cellular level. Furthermore, we analysed the influence of the tobamoviral 30K movement protein on AbMV, as the 30K protein was shown to have a capacity to support the spread of unrelated viruses (Giesman-Cookmeyer et al., 1995).

In symptoms and tissue distribution, AbMV resembles important tomato begomoviruses such as Tomato yellow leaf curl virus (Morilla et al., 2004). Its interactions with tobamoviruses were studied in Nicotiana benthamiana Dom in and in Lycopersicon esculentum Mill. ‘Moneymaker’ under controlled greenhouse conditions. Homozygous ToMV 30K movement protein-expressing tomato plants (cv. ‘Craigella GCR26’; Weber & Pfitzner, 1998; Weber et al., 1992) and non-transgenic controls were included in the experiments. Symptom development was quantified by optical rating and plant height measurements. Viral DNA or RNA accumulation was analysed by blots of total nucleic acid preparations from individual leaves of defined size and position on the plants (AbMV- or tobamovirus-specific probes). Whereas TMV and ToMV infected all leaf-cell types readily, exiting from the phloem in systemically invaded sink leaves (Cheng et al., 2000), tissue specificity of AbMV was determined by in situ hybridization (Morilla et al., 2004; Zhang et al., 2001).

In total, 284 N. benthamiana plants in four independent experiments were agroinfected with AbMV DNA A and B by stem pricking (Evans & Jeske, 1993; Frischmuth et al., 1993; Klinkenberg et al., 1989) or mock-inoculated using water or DNA B inoculum. Four to six days post-agroinoculation, they were superinoculated mechanically with TMV vulgare strain, ToMV (Meshi et al., 1986) virion preparations or buffer as a control onto two adjacent leaves above and below the agroinoculation site [60–70 (except for ToMV; n=16) plants per virus combination]. In preliminary experiments, simultaneous inoculation was also tested. With L. esculentum ‘Moneymaker’, the inoculation scheme of 126 plants (20–25 per combination, three experiments) was almost identical to that of N. benthamiana: stem agroinoculation was directed into cotyledon axils of seedlings with not yet unfolded primary leaves, which, after expansion, were inoculated mechanically with tobamovirus or buffer 5–7 days later. With ToMV 30K-expressing or non-transgenic control L. esculentum ‘Craigella’, two experiments were carried out (at least five plants each were mock- or AbMV-inoculated).

In both host species, 2–3 weeks post-agroinoculation, mixed infections had produced combinations of symptoms typical for either of the viruses with additional new phenotypic alterations (Fig. 1a; supplementary data in JGV Online). In N. benthamiana, AbMV or tobamoviruses alone caused mainly leaf symptoms, from leaf-blade curvatures to curling and chlorotic patches; the tobamoviruses also caused necrotic lesions on leaves and stem stunting, both more pronounced with ToMV than with TMV (data not shown). The combination of either tobamovirus with AbMV clearly enhanced stunting and the overall amount of necrosis. The rapid progress of symptoms obstructed exact height measurements, as doubly infected plants soon shrivelled from wilting and necrosis. Optical rating, however, showed a growth slowdown or arrest a few days post-tobamovirus superinoculation (p.i.) and indicated a synergism for the necrotic phenotype (not shown). Simultaneous agro- and mechanical inoculation of AbMV into stems and of tobamovirus onto pre-existing leaves lead to even more rapid disease development. As it frequently impeded unfolding of systemically co-invaded leaves, which we aimed to analyse, it was not used throughout the evaluated experiments. In tomato, leaf symptoms were similar to those in N. benthamiana except that in no case did necrosis occur, and not only green, but also yellow, mosaic developed, which was enhanced in the virus combinations. Most prominent, however, was a transient influence of double infection on plant height: none of the viruses alone induced significant stunting, but both viral combinations did (Fig. 1a). All sets of doubly infected tomato plants were stunted significantly compared with the singly infected ones up to about 25 days p.i. (i.e. 30–32 days after agroinoculation with AbMV, validated by t-test and Kruskal–Wallis analysis of variance on ranks). One to two weeks later, growth recovered. Thus, a truly synergistic, but transient, disease was produced whenever AbMV and either TMV or ToMV systemically co-infected a tomato plant, as was observed for N. benthamiana by the enhanced necrosis.

For N. benthamiana, this coincided with an unexpected reduction in susceptibility for AbMV by about 25 % when TMV was superinoculated [Table 1; with ToMV, 12 % fewer plants became infected, but the low number (16) did not suffice for statistical evaluation]. In N. benthamiana tissue explants, replicative forms of AbMV can be detected regularly as soon as 48 h post-agroinoculation (unpublished data). Therefore, it seems probable that tobamovirus inoculation into a different but nearby site of the plant, i.e. an adjacent leaf, resulted in a reduced efficiency of systemic infection by the geminivirus after its initial onset of replication. This may be due to an impeded multiplication or movement, but was not analysed in more detail. It might be speculated that RNA virus-mediated protection against a DNA virus could be triggered by the activation of host non-specific antiviral defences by the tobamovirus, resulting in begomovirus abortion in a subset of the plants [putatively involved systemic signalling pathways were reviewed by Beckers & Spoel (2006)]. Any similar RNA virus-induced protection against systemic AbMV infection was, however, absent in tomato.

To find out whether synergistic symptom enhancement was correlated with alterations of the begomovirus titre(s), blot hybridization experiments were carried out. Total nucleic acids were extracted from systemically infected (or control) leaves, or pinnate leaflets in the case of tomato, of all plants at different time points after infection by AbMV or TMV. Total nucleic acid preparations were divided into two samples each, with one sample used for the detection of AbMV and the other for the detection of TMV. The first set of samples was amplified with a primer pair that amplified all begomoviruses with the exception of TMV. The material from the second set of samples was amplified with a primer pair that amplified all tobamoviruses with the exception of AbMV. The RNA content of all samples was determined with a Qubit dsDNA HS Assay Kit (ThermoFisher Scientific). The size of the amplicons of both begomovirus and tobamovirus was determined by gel electrophoresis and verified by sequencing.

In summary, we analysed the influence of the tobamoviral 30K movement protein on AbMV, as the 30K protein was shown to have a capacity to support the spread of unrelated viruses (Giesman-Cookmeyer et al., 1995).
Young (not yet completely expanded) and mature, fully expanded leaves were compared (length of young versus mature leaves: *N. benthamiana*, up to 10 versus 15–20 mm; tomato, 50–80 versus about 200 mm). Samples containing equal amounts of plant genomic DNA were tested for relative levels of AbMV DNA and tobamovirus RNA in parallel on Southern or Northern blots, respectively (Fig. 2). Ethidium bromide

**Table 1.** TMV-mediated reduction in susceptibility of *N. benthamiana* for AbMV

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Inoculation</th>
<th>AbMV</th>
<th>TMV</th>
<th>AbMV + TMV</th>
<th>Infection by AbMV</th>
<th>Infection by TMV</th>
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<tr>
<td>1</td>
<td></td>
<td>30/30 (100)</td>
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<td>21/30 (70)</td>
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<td></td>
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<td></td>
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<td>7/7 (100)</td>
<td>4/7 (57)</td>
<td>7/7 (100)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>16/16 (100)</td>
<td>16/16 (100)</td>
<td>16/16 (100)</td>
<td>15/16 (94)</td>
<td></td>
</tr>
<tr>
<td>Mean (±sd)</td>
<td></td>
<td>98.5 (±3.1)</td>
<td>100 (±0)</td>
<td>74* (±18.3)</td>
<td>98.5 (±3.1)</td>
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* *t*-test: for z<0.05, AbMV infectivity is reduced significantly in comparison with singly AbMV-infected plants; *P*<0.038.
Fig. 2. (a) Southern (left panels) and Northern (right panels) blots indicating viral nucleic acid accumulation in *N. benthamiana* leaves. 1–24, Samples from individual plants 8/15 days p.i. (odd/even numbers). Equivalent amounts of plant genomic DNA were loaded (15 ng per sample; asterisks indicate exception lanes). Viral nucleic acid forms are indicated. (b) Median signal intensities (si) reflecting viral nucleic acid titre s in singly or doubly infected plants during progression of infection (four independent experiments, eight blots; ToMV-specific values following a similar course were excluded for reasons of clarity). Asterisk pairs indicate significant differences (α<0.05, Mann–Whitney rank sum test). (c) AbMV DNA in ToMV 30K-expressing and non-transgenic tomato (28 days p.i.).
staining of the corresponding agarose gels confirmed equal loading, and dilution series of AbMV- and TMV-derived hybridization standards confirmed reproducible band intensities between different blots, following probe detection via chemiluminescence and film exposure. All individual hybridization signals in equally loaded lanes were quantified (mean pixel intensities of identical areas; SigmaScan Pro, v. 5.0.0; SPSS Inc.) and used for statistical verification of differences in virus accumulation.

Typically, 75–100 % of the individual samples of same leaf age, time point of inoculation and infection status contained very similar levels of the respective viral nucleic acids. In *N. benthamiana*, accumulation of the begomovirus was repressed until at least 8 days p.i. upon co-infection with a tobamovirus (Fig. 2a, b). After a transient enhancement of AbMV DNA accumulation throughout the following week, it again declined, to remain reduced significantly compared with the single infections, from 18 days p.i. until the end of data collection (27 days p.i.) in both young and mature leaves. Calibration blots (Wege & Siegmund, 2007) allowed for the estimation that the diminished AbMV-specific hybridization signals at 18 days p.i. reflected viral DNA levels reduced by a factor of 10 (in young leaves) to 15 (in mature leaves). Contrasting with these observations, tobamoviral RNA titres in doubly infected plants in the early stages (8 days p.i.) were either indistinguishable from those in the single infections (TMV) or reduced (ToMV; Fig. 2a) but, after a period of suppression or delay, reached a peak of maximum accumulation between 16 and 18 days p.i. even more rapidly than in singly infected plants. Henceforth, they did not differ significantly from titres in single infections (Fig. 2b). All virus titres underwent an almost steady decline after 18 days p.i., reflecting the effects of ageing of the plants and necrogenesis.

For tomato, no systematic influence of mixed infection was observed at the level of virus accumulation (not shown). However, in 15–25 % of the doubly infected (but none of the singly infected) plants, begomovirus DNA titres were reduced transiently as in *N. benthamiana*, up to at least 15 days p.i. Two weeks later, AbMV accumulation had recovered.

Even if elevated virus titres can be excluded, a qualitative change in viral tissue distribution, e.g. the usually phloem-limited geminivirus BGMV dislocated into mesophyll upon tobamovirus co-infection (Carr & Kim, 1983), might serve as explanation for an increased pathogenicity. Therefore, leaf explants from highly symptomatic regions of several independent singly and doubly infected tomato plants were paraffin-embedded at 32 days p.i. and subjected to AbMV-specific *in situ* hybridization. However, the analyses of about 200 sections (each 2.5 mm in length) from different typical leaves co-infected with TMV or ToMV did not indicate an altered tissue tropism: all AbMV-specific signals were confined to cells associated closely with the phloem, as with the virus alone (Fig. 1b–d).

To establish whether the tobamovirus 30K movement protein by itself, in the absence of further viral RNA elements that might trigger antiviral defence in mesophyll tissues, might induce phloem escape of AbMV or be the responsible tobamoviral symptom determinant in the synergism, ToMV 30K protein-expressing tomato plants infected with AbMV were analysed for viral DNA content and tissue specificity. Neither the overall accumulation of AbMV DNA during the course of infection (Fig. 2c) nor its tissue tropism (Fig. 1e) was changed in these plants, compared with non-transgenic tomato of the same cultivar. Hence, the tobamoviral movement protein did not produce synergistic effects with a geminivirus in the common host.

The symptom-synergism determinants of the DNA and of the RNA viruses and their putative interacting partners in the family Solanaceae thus remain to be determined. The effects of enhanced pathogenicity on the one hand, and RNA virus-induced reduction of DNA virus accumulation on the other, differ from the former reports on tobamovirus–geminivirus interactions, but resemble those described for *Cauliflower mosaic virus* [CaMV; double-stranded (ds)DNA]–ssRNA virus interplay. Hii et al. (2002) detected symptom synergy of CaMV and the tobamovirus *Turnip vein clearing virus* in turnip (*Brassica rapa* L.), despite no change in the levels of either virus. Kamei et al. (1969) found that, in CaMV-preinfected *Brassica perviridis* Bailey plants challenged with the potyvirus *Turnip mosaic virus*, the latter was suppressed by the dsDNA virus. The negative effect may have been caused indirectly, due to impaired cellular functions as a consequence of disease. Similar reasons might also contribute to the negative interference between tobamoviruses and AbMV, but are unlikely to explain a transient suppression of virus accumulation in early stages of the infections or a reduction in susceptibility.

Molecularly, the most interesting aspect of this study is the failure of tobamoviruses to enhance AbMV replication and/or spread against the background of our contrasting findings for cucumoviruses. The synergism between CMV and AbMV was shown to involve the CMV 2b silencing-suppressor protein, which enhanced AbMV titres and numbers of invaded cells in different solanaceous hosts (Wege & Siegmund, 2007). Therefore, the inability of tobamoviruses to assist AbMV in the same host species might indicate that their respective RNA-silencing suppressors, i.e. the 126K or 130K proteins (Ding et al., 2004; Kubota et al., 2003), appear to be inefficient for AbMV infection, an observation that merits further investigation. Silencing-suppression activity has not yet been attributed to any AbMV protein and may implicate different begomoviral gene products (Bisaro, 2006). Therefore, the results may also suggest that AbMV differs from the geminiviruses BGMV, BCTV and TGMV in some of its strategies operating during tissue infiltration, as has been proposed previously (Zhang et al., 2001). The startling symptom synergism that we observed for the RNA–DNA virus combinations, which even induced completely new yellowing and stunting phenotypes, despite decreased virus accumulation, could be best explained by simultaneous
action of the two viruses on different host pathways, which in combination provokes an overall enhanced host response.

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References


