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RNA viruses and their silencing suppressors boost *Abutilon mosaic virus*, but not the Old World *Tomato yellow leaf curl Sardinia virus*

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ABSTRACT

Mixed viral infections can induce different changes in symptom development, genome accumulation and tissue tropism. These issues were investigated for two phloem-limited begomoviruses, *Abutilon mosaic virus* (AbMV) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) in *Nicotiana benthamiana* plants doubly infected by either the potyvirus *Cowpea aphid-borne mosaic virus* (CABMV) or the tombusvirus *Artichoke mottled crinkle virus* (AMCV). Both RNA viruses induced an increase of the amount of AbMV, led to its occasional egress from the phloem and induced symptom aggravation, while the amount and tissue tropism of TYLCSV were almost unaffected. In transgenic plants expressing the silencing suppressors of CABMV (HC-Pro) or AMCV (P19), AbMV was supported to a much lesser extent than in the mixed infections, with the effect of CABMV HC-Pro being superior to that of AMCV P19. Neither of the silencing suppressors influenced TYLCSV accumulation. These results demonstrate that begomoviruses differentially respond to the invasion of other viruses and to silencing suppression.

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1. Introduction

Invasion of a plant virus into new environments where other viruses are present is frequent. Agronomical, ecological and economical problems may result from increased symptoms (synergism), changes in virus accumulation, host range and vector transmission (Hammond et al., 1999; Latham and Wilson, 2008). Movement, capsid, or silencing suppressor proteins can be responsible for synergistic interactions as trans-complementing elements (Latham and Wilson, 2008).

Begomoviruses (family *Geminiviridae*) are emerging pests causing severe outbreaks with a relevant epidemiological potential worldwide, due to the wide distribution of their whitefly vector and the intercontinental transport of infected material. Their circular single-stranded (ss) DNA genomes, that multiply in nuclei, are organized in one or two molecules of about 2.7 kb each. Like most geminiviruses, *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and *Abutilon mosaic virus* (AbMV) are restricted to phloem cells (Horns and Jeske, 1991; Kim and Lee, 1992; Wang et al., 1996; Morra and Petty, 2000; Qin and Petty, 2001; Rojas et al., 2001; Morilla et al., 2004; Alves-Júnior et al., 2009), while few species also invade mesophyll or even epidermal cells (Wang et al., 1996; Morra and Petty, 2000; Wege et al., 2001). The ability to colonize non-vascular tissue does not simply correlate with the expression of DNA-B encoded transport-associated proteins (Zhang et al., 2001; Wege and Pohl, 2007; Kleinow et al., 2009), but may depend on different viral and plant factors, including non-coding nucleic acid sequences (Morra and Petty, 2000; Qin and Petty, 2001; Levy and Czosnek, 2003; Carvalho et al., 2006; Wege and Siegmund, 2007).

Mixed begomovirus infection led to synergistic interactions (Morilla et al., 2004; Chakraborty et al., 2008; Alves-Júnior et al., 2009), with possible changes in tissue tropism (Morra and Petty, 2000; Levy and Czosnek, 2003; Alves-Júnior et al., 2009). Studies on mixed infections by begomoviruses and viruses of other families are relatively scarce. Carr and Kim (1983) showed that co-infecting RNA viruses can alter the tissue specificity of a begomovirus. Accumulation of AbMV in *Nicotiana benthamiana* was increased upon co-infection by *Cucumber mosaic virus* (CMV), and several groups of contiguous cells adjacent to the phloem were detected to contain viral DNA (Wege and Siegmund, 2007). Transgenically expressed CMV 2b protein raised the number of AbMV-infected nuclei within the phloem. Conversely, tobamoviruses led to dramatic

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enhancement of AbMV symptoms but affected AbMV accumulation negatively and no phloem-escape was found (Pohl and Wege, 2007). To expand the knowledge on RNA–DNA virus interplay (Martín and Elena, 2009), we determined if potyviruses and tombusviruses, which synergistically interact with several RNA viruses (Latham and Wilson, 2008 and references therein), do so with begomoviruses as well. AbMV, chosen as a model of bipartite begomoviruses since it resembles important tomato-infecting New World begomoviruses in symptom induction and host invasion (Wege et al., 2001; Wege, 2007) was compared to TYLCSV, a member of the Old World viruses inducing the Tomato yellow leaf curl disease, causing severe epidemics not only in Eurasia, but also in an increasing number of American countries since the 1990s (Lefeuvre et al., 2010). The DNA viruses' molecular reactions towards the potyvirus *Cowpea aphid-borne mosaic virus* (CABMV) and the tombusvirus *Artichoke mottled crinkle virus* (AMCV) were analyzed. Both CABMV and AMCV express potent and well characterized RNA silencing suppressors, HC-Pro and P19, respectively (Mlotshwa et al., 2002a; Silhavy et al., 2002; Voinnet et al., 1999). We therefore compared the results of mixed infections with those obtained with *N. benthamiana* plants transgenically expressing CABMV HC-Pro (Mlotshwa et al., 2002a) or AMCV P19 (Silhavy et al., 2002). Symptom development, virus accumulation and tissue localization were monitored under different infection conditions.

2. Materials and methods

2.1. Plants and viruses

N. benthamiana non-transgenic (nt) or transgenic for CABMV-Z HC-Pro (line h-44, Mlotshwa et al., 2002a), or for AMCV P19 (line A30, Silhavy et al., 2002) were grown in greenhouse at 20–28/16–20 °C (day/night), with a 16/8 h (light/dark) photoperiod with supplementary lighting.

Begomoviruses were delivered to seedlings at the 2–4 leaf stage by stem inoculation of *Agrobacterium tumefaciens* LBA4404 cells carrying DNA-A and -B clones of AbMV (AbA and AbB, GenBank Acc. No. X15983/X15984; Frischmuth et al., 1993) or TYLCSV (X61153; Kheyr-Pour et al., 1991). Both the potyvirus CABMV-Z (X82873) and the tombusvirus AMCV (X62493) were propagated in *N. benthamiana* plants, and homogenates of systemically infected leaves from frozen stock tissue were used for mechanical inoculation.

To establish double infections, plants were inoculated first with either begomovirus and 4 days later with either AMCV or CABMV-Z. Reference plants inoculated with a single virus were mock-inoculated instead of treatment with the second virus, using solely AbMV DNA-B or an empty pBIN19 vector containing *Agrobacterium* instead of begomoviruses, or the inoculation buffer in place of an RNA virus.

2.2. Analysis of transgene-mediated silencing-suppression

Seedlings of the A30 and h-44 lines were tested for the presence of transgenes by PCR (primers listed in Supplemental Table 1) on total DNA extracted from individual seedlings: [30 s at 94 °C, 40 s at 50 °C and 2 min at 72 °C] (40 cycles) for line h-44; and [30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C] (35 cycles) for line A30. To test expression of the functional transgenes, fully expanded leaves were infiltrated with *A. tumefaciens* LBA4404 carrying a 35S-GFP plasmid (provided by Prof. D. Baulcombe, Cambridge, UK). Fresh colonies were grown overnight in YEB medium (containing 100 μg/ml kanamycin, 50 μg/ml rifampicin and 40 μM acetosyringone; 28 °C, 200 rpm), centrifuged (6000 × *g*, 15 min) and re-suspended in infiltration buffer [10 mM 2-(N-morpholino)ethane sulfonic acid (MES) buffer, pH 5.6, 10 mM $MgCl_2$, 200 μM acetosyringone] to an OD_{600} = 0.8. After a 3-h incubation at room temperature, bacteria were infiltrated using a 1-ml syringe without a needle. GFP expression was monitored under UV light (365 nm; UVP lamp -B-100AP, 100 W, Upland, USA) and by western blot analysis of total proteins [SDS-15% polyacrylamide gels/PVDF membranes; monoclonal antiGFP antibody; SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA)].

2.3. Symptom and biomass analysis

Plants were scored for symptom development in 17 parallel experiments during a time course up to 45 days post agroinoculation/41 days post mechanical inoculation (45/41 dpi). Above ground biomass of 10 individual plants per treatment was measured at 45/41 dpi with an analytical balance (drying conditions 80 °C for 3 days) for a representative experiment, and variance (ANOVA) was determined using the Fisher's least significant difference (LSD) post hoc test (p = 0.05).

2.4. Viral nucleic acid detection

2.4.1. DNA viruses

Total nucleic acids (TNA) extracted from young leaves (10–20 mm long; Wege and Siegmund, 2007) were quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). Five-hundred nanograms of TNA per lane were analyzed in Southern blots. For AbMV, membranes were hybridized with either a full length DNA A probe (Wege and Siegmund, 2007) or a CP gene-specific one amplified from AbMV-infected DNA extracts using primers GEM-AV494 and GEM-AC1048 (see Supplementary Table 1), as specified in figure legends, with no detectable differences in sensitivity or specificity. For TYLCSV, a CP gene-specific probe was used (see Supplementary Table 1).

2.4.2. Semiquantitative blot evaluation

Equal loading of plant DNA in each lane was confirmed by ethidium bromide staining of the agarose gels. Semiquantitative rating of relative begomoviral DNA amounts followed probe detection via chemiluminescence and film exposure using AbMV- or TYLCSV-derived hybridization standards (1 pg to 1 ng) as reference. In order to determine the relation of hybridization signal densities to viral DNA amounts, 'calibration blots' (Wege and Siegmund, 2007) with serial dilutions between 0- and 1000-fold (representing 10 μg to 10 ng TNA) were used. Pairs of singly and doubly infected, or nt and transgenic plants were chosen at random, and the differences in the relative viral DNA amounts were assessed for the sum of virus-specific signals present in each lane on the same blots. Visual inspection was verified by quantification of mean pixel intensities for identical areas using image analysis software (SigmaScan Pro, v. 5.0.0; SPSS Inc.).

2.4.3. RNA viruses

All samples were tested for the presence of co-invading RNA viruses. Total RNA was extracted using TRIzol (Invitrogen) and applied to dot blots. Membranes were hybridized using digoxigenin-labeled DNA probes specific for CABMV or AMCV obtained by PCR using TNA templates from h-44 or A30 plants, respectively, and specific primers (Supplementary Table 1). A probe specifically detecting the *N. benthamiana* 26S ribosomal RNA (Supplementary Table 1) was used to ensure equal RNA loading.

2.5. Viral nucleic acid detection by in situ hybridization (ISH)

Localization of AbMV and TYLCSV DNA in plant tissues was visualized by ISH on aldehyde-fixed sections. Specimens were obtained from explants (5 mm × 10 mm) at 25/21 and 31/27 dpi for mixed

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