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Synergism of a DNA and an RNA virus: Enhanced tissue infiltration of the begomovirus *Abutilon mosaic virus* (AbMV) mediated by *Cucumber mosaic virus* (CMV)

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Abstract

Replication of the begomovirus *Abutilon mosaic virus* (AbMV) is restricted to phloem nuclei, generating moderate levels of virus DNA. Co-infection with *Cucumber mosaic virus* (CMV) evidently increased AbMV titers in *Nicotiana benthamiana*, tobacco, and tomato, resulting in synergistic symptom enhancement. *In situ* hybridization revealed that in double-infected leaves an increased number of nuclei contained elevated amounts of AbMV. Additionally, the begomoviral phloem-limitation was broken. Whereas CMV 3a movement protein-expressing tobacco plants did not exert any similar influence, the presence of CMV 2b silencing suppressor protein lead to enhanced AbMV titers and numbers of infected vascular cells. The findings prove that AbMV can replicate in nonvascular cells and represent the first report on a true synergism of an RNA/ssDNA virus combination in plants, in which CMV 2b protein plays a role. They indicate considerable consequences of mixed infections between begomo- and cucumoviruses on virus epidemiology and agriculture.

Keywords: Geminivirus; Begomovirus; *Abutilon mosaic virus*; Cucumovirus; *Cucumber mosaic virus*; Synergism; *In situ* hybridization; 2b silencing suppression; Phloem tropism; Tomato

Introduction

Legumes, cucurbits, and solanaceous crops are cultivated worldwide. They are of vital economic importance for many of the countries growing them, and they are prone to being diminished or regionally devastated by epidemics caused by begomoviruses or cucumoviruses. Increasing numbers of agronomically relevant outbreaks of diseases caused by circular ssDNA-containing begomoviruses (family: *Geminiviridae*) have been reported throughout the last decades (Brown and Bird, 1992, and references herein; Legg and Fauquet, 2004; Moriones and Navas-Castillo, 2000; Polston and Anderson, 1997; Ribeiro et al., 2003; Rybicki and Pietersen, 1999). By comparison, the plus-stranded ssRNA *Cucumber mosaic virus* (CMV; family: *Bromoviridae*) is endemic due to its very broad host range of more than 1200 plant species (Palukaitis and García-Arenal, 2003a), many of which are also susceptible to infection by geminiviruses. Both virus groups share not only hosts, but other properties: their outstanding evolutionary success (Mansoor et al., 2003; Roossinck, 2002) is, for most begomoviruses, associated with bipartite, or in the case of cucumoviruses, tripartite, segmented genomes conferring the capacity of forming reassortants (pseudorecombinants). There is also clear evidence of ecologically relevant proportions of viruses harboring physically recombined new genome molecules which, under conditions of selection pressure, may exhibit raised fitness and epidemiological relevance (Aaziz and Tepfer, 1999; Ariyo et al., 2005; Bonnet et al., 2005; García-Arenal et al., 2001; Monci et al., 2002; Pita et al., 2001). Furthermore, both viral genera have the ability of supporting satellites or satellite-like subviral molecules which are widespread and diverse, and can cause new phenotypes (Briddon et al., 2003, 2004; Briddon and Stanley, 2006; Roossinck et al., 1992). So

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both virus groups have to be regarded as rather flexible disease complexes than defined pathogens whose impact on worldwide agronomy is high and still increasing (Mansoor et al., 2003; Simon et al., 2004; Stanley, 2004). As a consequence thereof, those viruses co-infect crop and wild plants frequently, which we sought to address by a model study reported here.

Viral interplay was monitored in laboratory hosts (*Nicotiana benthamiana* and *Nicotiana tabacum*) as well as in tomato by use of the bipartite begomovirus *Abutilon mosaic virus* (AbMV; Frischmuth et al., 1990; Wege et al., 2000) and two CMV strains. Genome structures, infection strategies, and protein functions of geminiviruses and cucumoviruses have been analyzed in detail (Palukaitis and García-Arenal, 2003b; Palukaitis et al., 1992; Stanley et al., 2005). Begomoviral proteins are expressed bidirectionally from dsDNA intermediates of singly encapsidated circular DNAs; their functions include cell-cycle manipulation, suppression of host defense/posttranscriptional gene silencing (PTGS), and intra- as well as intercellular trafficking of nucleoprotein-complexes (Bisaro, 2006; Hehnle et al., 2004; Rojas et al., 2005; Stanley et al., 2005; Zhang et al., 2001). AbMV in symptom formation and in its strict phloem-limitation (Wege et al., 2001) resembles important tomato begomoviruses like *Tomato yellow leaf curl virus* (TYLCV), which is known to be responsive to the presence of additional viruses with respect to disease induction (Morilla et al., 2004). By contrast, both CMV strains used in this study efficiently infiltrate mesophyll and spread throughout almost every cell of systemically infected leaves (Cillo et al., 2002; Takeshita et al., 2004b). The tripartite, capped (+)ssRNA genome of CMV is packaged into at least three separate particles in combination with subgenomic RNAs. Among other proteins, the CMV genome also encodes a PTGS suppressor (protein 2b) and the movement protein (MP) 3a (Palukaitis and García-Arenal, 2003b).

Numerous simultaneous infections with distinct viruses have been observed in the natural situation (Rochow, 1972) and may result in unpredictable effects from disease amelioration up to symptom synergy (Hammond et al., 1999). Plant viral double-infections which have been analyzed in detail so far comprise mainly combinations of different ssRNA viruses, frequently including poty-, tobamo-, and cucumoviruses (Atabekov et al., 1999; Hull, 2002; Malyshenko et al., 1989; Martin et al., 2004; Palukaitis and García-Arenal, 2003b). In studying combinations of gemini- and cucumoviruses with respect to symptoms, virus accumulation, and tissue tropism at high resolution, we have characterized an interaction between plant RNA and DNA viruses at the molecular and cellular levels for the first time. Plant and leaf growth kinetics were taken into account. As MPs of RNA viruses had been shown to support spread of unrelated viruses (Waigmann et al., 2004), and since transgenically expressed CMV 3a protein was able to mediate plasmodesmata gating and cell-to-cell transport of heterologous or movement-defective viruses (Ding et al., 1995; Kaplan et al., 1995; Vaquero et al., 1994), plants expressing functional CMV 3a MP were included in the experiments. Increasing evidence also points to important roles of viral silencing suppressor proteins in inducing synergistic disease and defining tissue invasion patterns in systemic single or mixed virus infections (e.g. Bisaro, 2006; Roth et al., 2004; Scholthof, 2005, and references therein). Therefore, particularly detailed analyses were carried out on two further tobacco plant lines, both transgenic for CMV 2b.

Results

AbMV in double-infection with CMV results in symptom enhancement

In comparison to the single infections, co-infection of *N. benthamiana*, *N. tabacum* cv. Samsun nn, or NN, and tomato plants with AbMV and either CMV strain (Fny or Le) resulted in increased systemic symptoms (Fig. 1; *N. tabacum*: data not shown). Symptoms with Fny-CMV generally were more severe than with Le-CMV, with the exception of AbMV-/Le-CMV-infected tomatoes (see below). Both CMV strains typically induced stunting, mosaic, yellowing, leaf lamina wrinkling, and deformation, in tobacco and especially in tomato also leaf blade reduction. In newly developed leaves of both tobacco and tomato, cyclical symptom ameliorations occurred as has been described by Gal-On et al. (1995). AbMV alone induced a relatively mild phenotype in all of the hosts, comprising mild stunting, weak leaf rolling or distortion and inconspicuous green mosaic in *Nicotiana* sp., or yellow-green mosaic and leaflet wrinkling in the absence of stunting in tomato (Fig. 1B). Double-infected plants in all cases combined the symptoms typical for both of the viruses, but also developed additional leaf deformations and yellowing. With respect to biomass, in the case of *N. benthamiana* statistically significant synergistic responses were observed during disease progression. Since co-inoculation of RNA and DNA viruses lead to rapid death of the plants before viral effects could be quantified, mechanical inoculation of CMV was carried out 6 to 12 days post agroinfection (dpai) in all analyses described here. Figs. 1A and B (Experiment 1) represent a typical set of plants: The fresh weight of doubly infected *N. benthamiana* was reduced synergistically: AbMV/Le-CMV plants exhibited 42% reduction in contrast to 19% expected for an additive effect; AbMV/Fny-CMV plants showed a reduction of 65% in contrast to the expected 57%. For the plants' biomass (dry weight), synergism was observed for AbMV/Le-CMV (50% reduction observed/43% expected), whereas AbMV/Fny-CMV produced an additive reaction (70% reduction): For those highly affected plants, the period of measurable synergism was over. Fig. 1B shows an independent experiment (no. 2) at a late stage (63 dpai) when infections had proceeded too far to still detect synergism: AbMV and Le-CMV had led to significant growth reduction by themselves, and Fny-CMV had induced extensive or complete necrosis of its hosts.

With tomato, a complex disease time course was observed, reflecting the cycling effects of CMV on its own. Periodic symptom synergism occurred with AbMV plus either CMV strain, but was most pronounced with Le-CMV. In single infection with Le-CMV, after one or a few cycles of strong symptoms and consecutive recovery, plant death was occasionally induced. In the double-infections, severe stunting resulted in

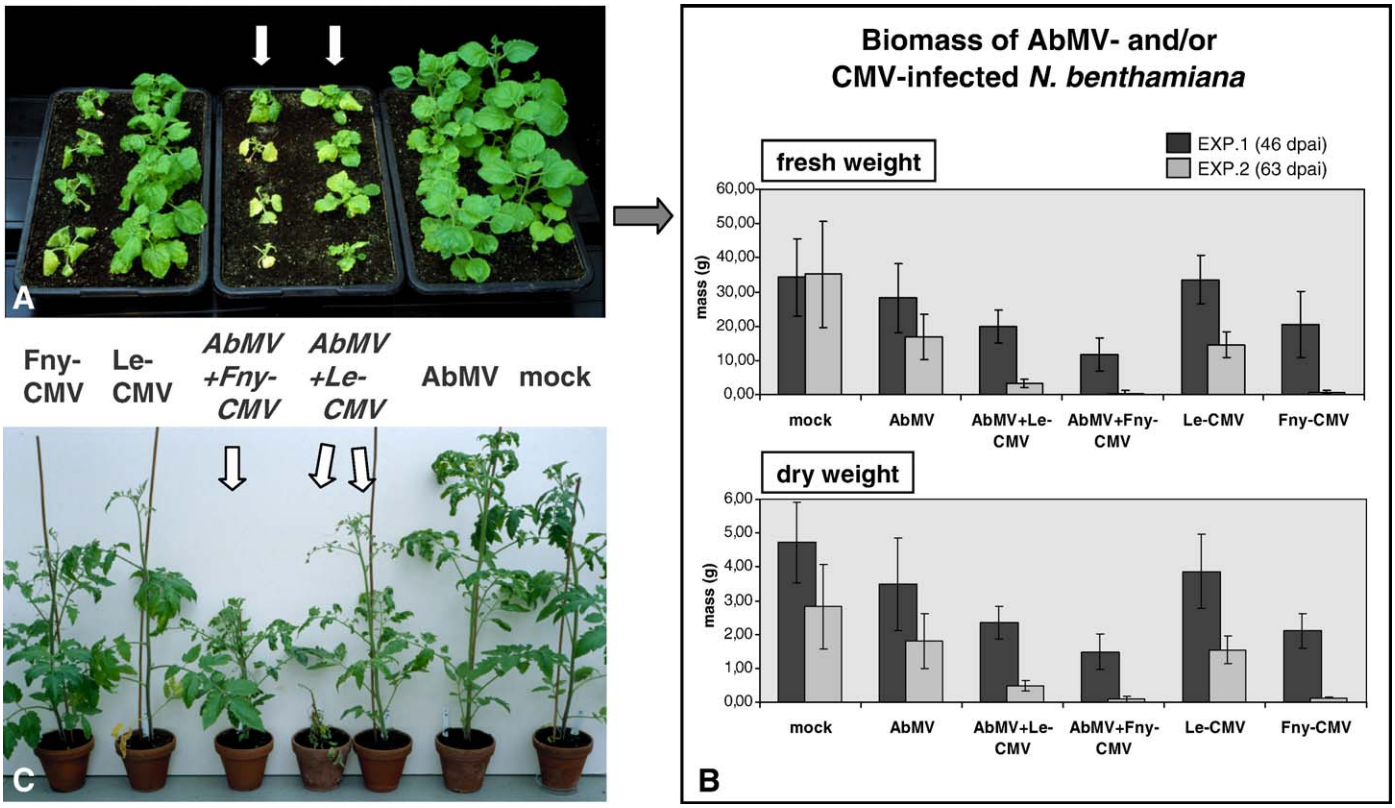

Fig. 1. AbMV in single and in co-infection with CMV in *N. benthamiana* (A, B) or in tomato plants (C). (A) *N. benthamiana* singly or doubly infected as indicated, 46 days post agroinfection (dpai). (B) Biomass of plants shown in A (experiment 1; 46 dpai), and of another experiment (experiment 2) analyzed at 63 dpai, both according to fresh or dry weight, respectively. Infection of plants from experiment 2 had proceeded too far to still exhibit synergistic effects, which are obvious for experiment 1. (C) Representative tomato plants infected with the virus(es) indicated, at 36 dpai. Note that an Le-CMV-infected tomato plant has died from apical necrosis.

apex necrosis more frequently. Stem height measurements revealed that strongly accelerated disease periods (very prominent four to five weeks post agroinoculation (wpai), Fig. 1C) alternated with periods of recovery for the few surviving Le-CMV/AbMV-infected tomatoes, and for all the Fny-CMV/AbMV-infected plants over several months. True synergism four to five wpai typically lead to plant height reduction by about 35% or 45 to 50% with Fny-CMV or Le-CMV, respectively, in co-infection with AbMV (compared to the absence of any significant reduction with Le-CMV or AbMV alone, and a reduction of about 10% with Fny-CMV). Similar observations were made with *N. tabacum* cv. Samsun NN plants, Fny-CMV being a bit more effective than Le-CMV in this host (at 6 wpai stunting either did not occur or was very mild with CMV, and plant heights were reduced by 15% with AbMV alone, by 35% with Fny-CMV/AbMV and by 30% with Le-CMV/AbMV). CMV-caused leaf symptoms exhibited cycling as in tomato.

Double-infected plants accumulate increased levels of geminivirus DNA

AbMV DNA accumulated to significantly higher titers in double-infections in all host species and with both CMV strains used, as revealed by Southern blot analyses of total nucleic acids isolated from single leaves or pinnate leaflets. In those respective samples, the amount of all viral DNA forms, including double-stranded (ds) intermediates (for details, refer to Jeske et al., 2001) and single-stranded (ss) DNA, increased. In *N. benthamiana*, the effect was prominent in every leaf analyzed (Fig. 2). An estimation of relative AbMV DNA quantities by use of standardized blots indicated that the titer of viral DNA was typically elevated by a factor of five to 15 (data not shown). On the other hand, enzyme-linked immunosorbent assay (ELISA) revealed for all hosts that CMV concentrations did not significantly differ from those in plants infected by CMV alone (data not shown). Therefore, the synergy between CMV and AbMV involves one virus enhancing the symptoms and the titer of the other. In the case of tobacco and tomato, only about one third of all individual leaves (or leaflets) of mixed-infected plants analyzed on blots contained clearly about two- to tenfold increased amounts of AbMV DNA in comparison to the singly infected controls, irrespective of the developmental stage of the plant or the leaf. This is exemplified in Fig. 3, showing blot analyses of individual leaflet samples from tomato plants at different stages of development, collected from a set of ELISA-validated plants at 29 and at 46 dpai (when some plants had died). However, from almost any of the co-infected plants, at least one leaflet at one point of time contained higher amounts of AbMV DNA. As in *N. benthamiana*, all forms of viral DNA appeared to be amplified with no change in the CMV titer. The presence of all three RNA segments of CMV

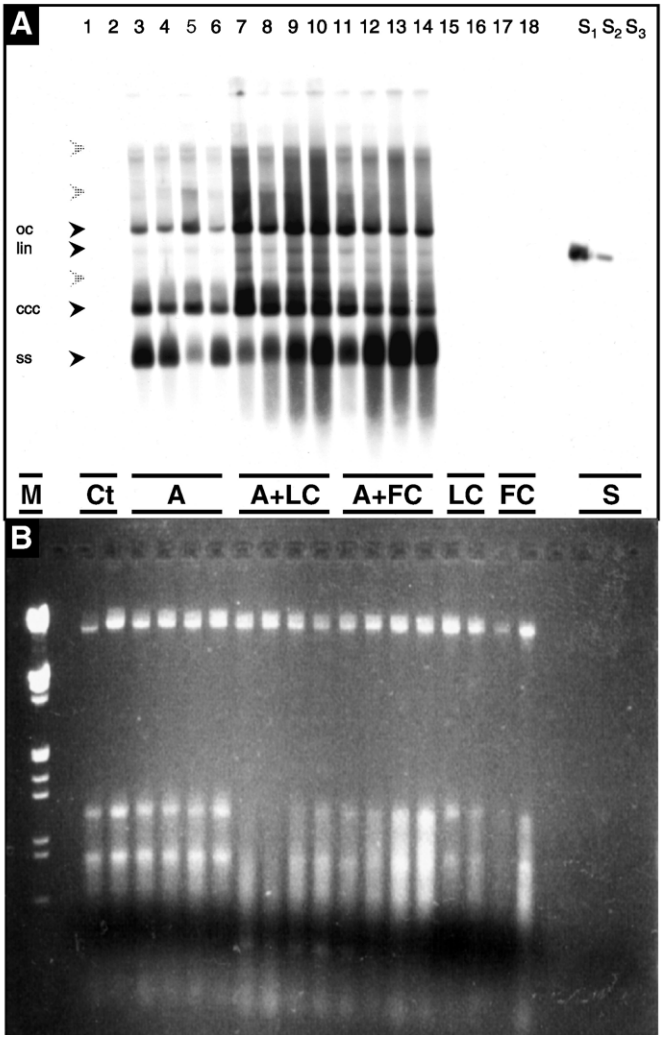

Fig. 2. Relative AbMV DNA amounts in singly and doubly infected *Nicotiana benthamiana* as shown by Southern blot analysis (A). (B) Corresponding 0.8% agarose gels run in the presence of ethidium bromide (0.5 μg/ml). Sample loading as indicated (A: AbMV, LC: Le-CMV, FC: Fny-CMV); S: Standards for hybridization S_1 to S_3 (linear AbMV DNA A; 100/10/1 pg). Ct: Control plant (mock-inoculated). M: Marker in panel B is Lambda DNA digested with *Eco*RI/*Hin*dIII. Main AbMV DNA forms are indicated by arrows, black: oc or lin: open circular or linear circular dsDNA, ccc: covalently closed circular dsDNA, ss: single-stranded DNA, grey: di- or multimeric and heterogeneous DNA.

was confirmed by Northern blot hybridization of selected samples (data not presented). In *N. tabacum*, a similarly increased CMV-dependent accumulation of AbMV DNA occurred, but only transiently in very young leaves, which differed from the high AbMV DNA titers in several co-infected tomato leaves (Figs. 4A, B, compare with Fig. 3B, and to a lesser extent Fig. 3D).

An elevated number of AbMV-containing nuclei and an altered tissue tropism in doubly-infected leaves

To determine the numbers of infected cells and to analyze the tissue tropism of AbMV in single and in mixed infections with CMV, *in situ* hybridization was carried out on a large number of sections from systematically embedded singly and doubly infected leaf specimens of *N. benthamiana* and tomato. Whereas CMV infiltrates all tissues and cell types of leaves (Cillo et al., 2002; Takeshita et al., 2004b), AbMV so far has been localized exclusively to the phloem of different host plants (Horns and Jeske, 1991; Wege et al., 2001). Fig. 5 shows that, in this study, the previous results were again confirmed for singly AbMV-infected *N. benthamiana* leaves of different ages (Figs. 5B, C). In co-infection with Fny- as well as with Le-CMV (Figs. 5D to F), however, significant changes in AbMV distribution were observed: *In situ* localization of viral DNA in specimens from strongly affected and thus typically synergistically infected leaves revealed that AbMV DNA was frequently present in nuclei of neighboring cells, which was only occasionally observed in singly AbMV-infected *N. benthamiana* analyzed in parallel. In completely chlorotic co-invaded young leaves, an intriguing novel AbMV distribution was found: In co-infection with Le- or Fny-CMV (as represented for Fny-CMV by Figs. $5F_{1-4}$), the otherwise strictly phloem-limited begomovirus reproducibly had penetrated palisade or spongy parenchyma cells at sites close to tissue sectors harboring numerous AbMV-containing cells next to each other. Six out of 24 slides with specimens from young doubly invaded leaves each exhibited several phloem-escape sites. The newly gained competence of mesophyll infiltration was accompanied by a significantly increased number of infected cells, as illustrated in Table 1 (two- to five-time increase validated by Mann–Whitney Rank Sum Test on median values of mean numbers of hybridization signals per slide, counted on more than 500 *N. benthamiana* sections), and by intensified individual hybridization signals indicative of an elevated intranuclear titer of virus DNA. In contrast to those young leaves up to about 1 cm in length, larger leaves of at minimum 2 cm did not yield the same observations. This may be explained by the fact that during the synergistic period, growth was slowed down to such an extent that none of the strongly affected leaves ever reached the size and developmental stage of the respective control plants' leaves of similar age, and thus all "mature" samples were derived from early invaded, old leaves.

Tomato plants, as described above, also developed synergistically enhanced symptoms when simultaneously infected with AbMV and CMV, including reduction in leaf blade area and stunting which followed a complex cyclical time course. The phenotype was accompanied by elevated amounts of AbMV DNA within 30% of the sampled tomato leaflets of different ages. In order to correlate those findings with virus spread, the tissue tropism of AbMV alone was determined for tomato in this study, and compared to that in CMV-co-infected plants (Figs. 6A, B). AbMV by itself was closely associated with the phloem, similar to its distribution in all other hosts analyzed before (*Abutilon* sp., *Malva parviflora*, *N. benthamiana*, *N. tabacum*; Horns and Jeske, 1991; Wege et al., 2000, 2001). The total number of AbMV DNA-harboring nuclei per mm leaf blade section was, however, significantly higher than in other species (about four times that of *N. benthamiana*, see Table 1), concomitant with inner and outer phloem sectors

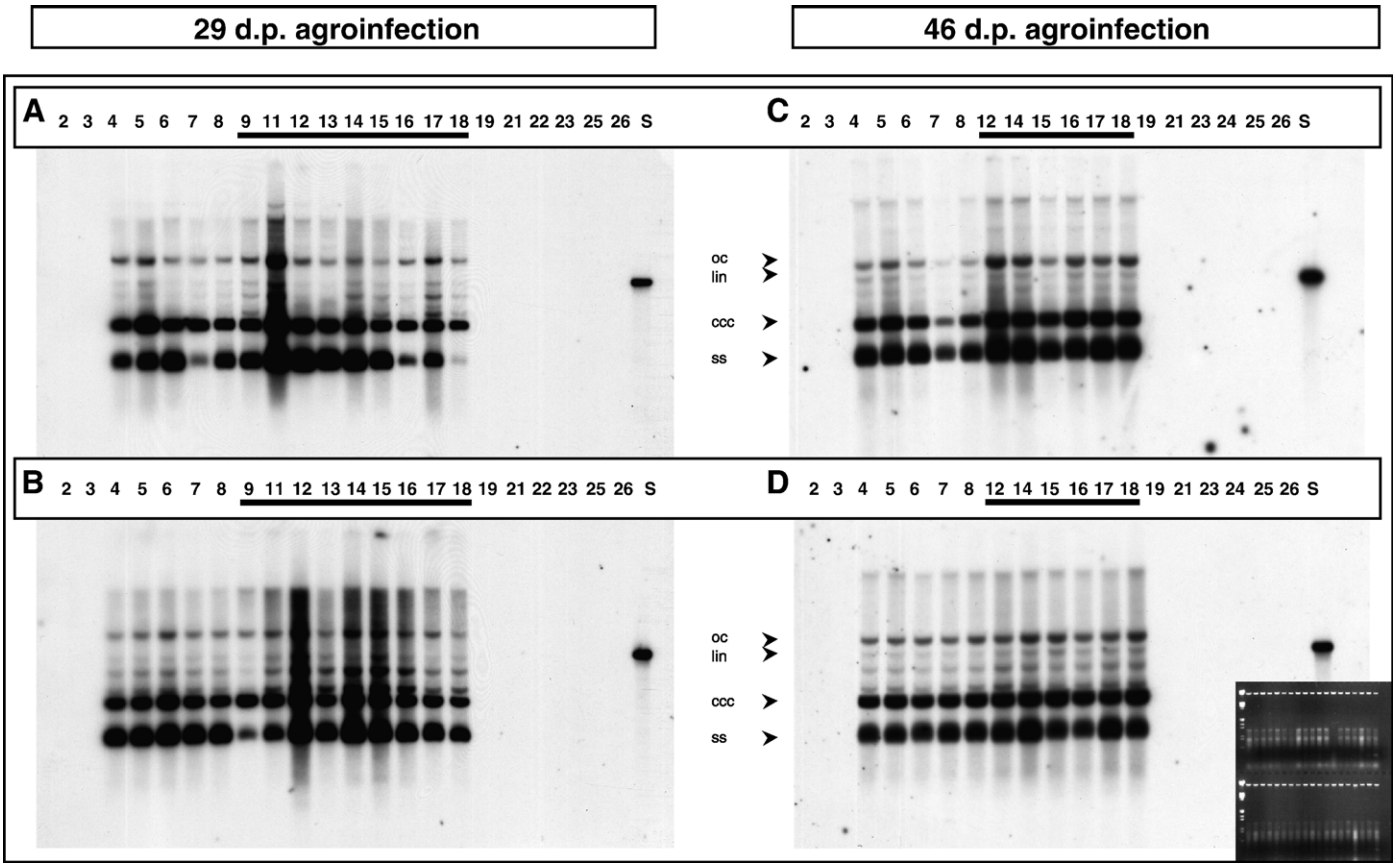

Fig. 3. Relative AbMV DNA amounts in singly and CMV co-infected tomato plants in leaves of different ages at different dates post agroinfection. Lanes represent individual plants: 2, 3: mock-inoculated, 4–8: AbMV singly infected, 9–13: AbMV/Le-CMV-infected, 14–18: AbMV/Fny-CMV-, 19–22: Le-CMV-, 23–26: Fny-CMV-infected. CMV infections were confirmed by ELISA; missing numbers indicate unsuccessful infection or death of plant. (A, B) Samples harvested 29 dpai, (C, D) samples from 46 dpai (A, C: pinnate middle leaflets from young, or B, D: from fully developed third to fifth leaves, respectively). Samples containing equal amounts of plant genomic DNA were separated on 0.8% agarose gels containing EtBr, blotted and hybridized as described in the text. S: Standard, linear AbMV DNA A (100 pg). AbMV DNA forms indicated by arrows as described for Fig. 2. Note that, except for plant no. 13 which died, in all of the plants double-infected with either of the CMV strains and AbMV, at least one leaflet at one point of sampling time contained elevated titers of AbMV DNA. Inlay in panel D shows gel A/B.

containing several adjacent AbMV-infected nuclei (Fig. 6A_2). In doubly infected tomatoes exhibiting increased symptoms, the situation was clearly changed, by analogy to the observations in *N. benthamiana*: In six out of 20 slides analyzed, AbMV DNA had unequivocally entered mesophyll cell layers at several sites such as palisade parenchyma, far distant from the vascular tissue (Fig. 6B). No similar distribution pattern was observed in 14 slides with singly AbMV-infected specimens. In contrast to the findings for *N. benthamiana*, however, median numbers of infected cells per mm specimen were not significantly altered in the mixed infections. This may be attributed to a very heterogeneous distribution of AbMV between different leaf blade sectors on the one hand (every slide with 12 consecutive sections covers only about 2.5 × 0.1 mm leaf lamina), and the low proportion of only one third of the samples with clearly raised overall AbMV DNA amounts on the other. Therefore, those leaves or leaf areas with altered tissue tropism *and* increased numbers of nuclei may not have been selected in our random sampling. These findings prove, however, that mesophyll invasion is not necessarily dependent on locally increased numbers of infected cells.

The CMV 3a movement protein does not potentiate the spread of AbMV

Since the tissue infiltration of AbMV was enhanced upon co-infection with CMV, we sought to determine whether the CMV MP itself could support geminiviral movement. To this aim, we included transgenic tobacco expressing a functional Fny-CMV 3a protein in our studies, which had been shown to complement cell-to-cell transport of some related viruses deficient in local movement (Kaplan et al., 1995). AbMV induced the same stunting in the transgenic *N. tabacum* cv. Turkish Samsun NN plants exactly as in the nontransformed controls (20% reduction in size at late stages, 7 wpai, compared to mock-inoculated plants). Moreover, no alteration in AbMV leaf symptoms was observed. Fig. 4 illustrates that begomovirus DNA amplification occurred in the transgenic plants (lanes 13 to 17) indistinguishable from that in nontransformed tobacco (lanes 1 to 4). At least in the young leaves, this differs from the result obtained for plants co-infected with AbMV and either Le- or Fny-CMV. At least two different leaves each from 15 independent transgenic plants in two experiments were tested that way, either at 32 or 42 dpai, and none of the samples

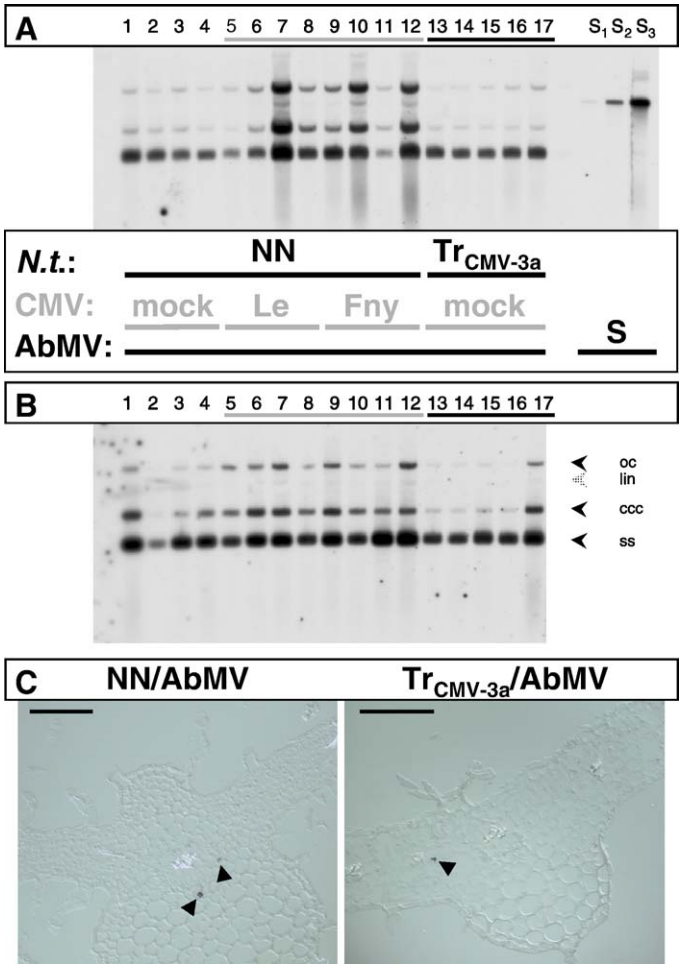

Fig. 4. Comparison of relative AbMV DNA A titers in individual *N. tabacum* cv. Samsun NN plants infected with AbMV alone (plants 1–4), in mixed infection with CMV (plants 5–12, CMV strain as indicated), and in transgenic *N. tabacum* cv. Samsun NN plants expressing the Fny-CMV 3a protein (plants 13–17; A, B) with corresponding *in situ* localization (C), 6 wpai. Panels A and B: Southern blot analysis of total nucleic acid samples from individual tobacco leaves (A: young leaves, up to 1 cm in length; B: larger leaves, 6–8 cm), using equal amounts of genomic plant DNA per lane. S: Hybridization standards as in Fig. 2, 1 to 100 pg. (C) Detection of AbMV DNA in vascular tissues (dark stain) following *in situ* hybridization of leaf sections (plant type as indicated; arrowheads point at signals). Differential Contrast Microscopy (DIC). Scale bars

exhibited increased AbMV DNA titers. *In situ* hybridization yielded clear evidence that AbMV accumulation was limited to a low number of phloem nuclei, irrespective of whether CMV-3a protein was present or not (Fig. 4C).

The CMV 2b protein is sufficient to enhance AbMV DNA titer and numbers of invaded cells

In order to test if suppression of plant antiviral silencing by the CMV 2b protein accounts for the observed effects, symptoms, accumulation and tissue tropism of AbMV were analyzed in two tobacco plant lines expressing 2b protein from stably integrated copies of full-length Q-CMV RNA 4a (Ding et al., 1994) under the control of the CaMV 35S promoter. Two sets of *N. tabacum* cv. Samsun nn plants, line C2b (Ji and Ding, 2001), and two sets of *N. tabacum* cv. Samsun nn x cv. Paraguay PBD6 plants, line C2bx6b5 (Elmayan and Vaucheret, 1996; Guo and Ding, 2002), were agroinfected with AbMV (or subsets mock-inoculated). The expression of the 2b transgene was repeatedly confirmed between 31 and 67 dpi in both hemizygous lines. Roughly 50% of the plants in both lines were shown to transcribe ORF 2b, as verified by RT-PCR (results not shown). For line C2bx6b5 which carries an additional β-glucuronidase (GUS) transgene, protein 2b-induced GUS activity was detected in leaf veins. The latter indicated the silencing suppressor's influence on the otherwise autonomously silenced GUS gene (as exemplified in Fig. 7; Guo and Ding, 2002). Using PCR, RNA 4A transgene was detected in two independent isolations of plant genomic DNA. Kanamycin resistance was checked in parallel (Fig. 7). Plants tested positive in all assays were taken for "CMV 2b expressors", whereas sibling plants in the same progeny which were negative in all assays, as well as nontransformed *N. tabacum* cv. Samsun nn plants, served as controls.

Compared to the nontransformed, plants in both transformed lines were growing slowly and heterogeneously, irrespective of whether they expressed 2b protein, were systemically infected with AbMV, or not. No significant difference was noticed between all AbMV-infected plant groups of different genetic background, and none of the plants developed any differential leaf symptoms, so no influence of CMV 2b protein on AbMV symptom induction was obvious. Furthermore, Southern blot analyses on total nucleic acids extracted from young or mature leaves did not reveal any obvious change in AbMV DNA levels in the 2b protein-expressing plants of both tobacco lines in comparison to the respective nontransgenic siblings up to 63 dpi, when plants were grown in a climate chamber under moderate conditions (day: 16 h 23 °C/night: 8 h 20 °C; Figs. 7A, D, and data not shown). At 85 dpi, all plants were transferred into conditions of high day temperature and light (day/night: 30/18 °C; supplemental lighting), and subjected to a further Southern analysis 10 days later. Figs. 7B, C, E, and F present clear evidence that under those altered environmental conditions, CMV 2b protein indeed mediated the accumulation of significantly increased amounts (by a factor of two to fifteen) of all AbMV DNA forms in the tobacco plant line C2bx6b5, but not in line C2b. Strikingly, viral DNA titers were evidently elevated not only in young leaves, as it was observed in doubly infected nontransgenic plants (Fig. 4), but also in almost any of the mature leaves tested (Fig. 7F).

To find out if CMV 2b-protein transgenic plants supported an enhanced tissue infiltration by AbMV, specimens from systemically invaded young and mature leaves of all plant types, and the respective uninfected controls, were paraffin-embedded at three timepoints, twice before and once two weeks after changing cultivation conditions. *In situ* hybridization was carried out on more than a thousand sections (refer to Table 2 for details). Plant line C2b, in comparison to nontransgenic siblings, showed no obviously altered AbMV accumulation inside leaf tissues at any time throughout the experiments (Table 2, and Fig. 8). Like in nontransformed wild type tobacco, the

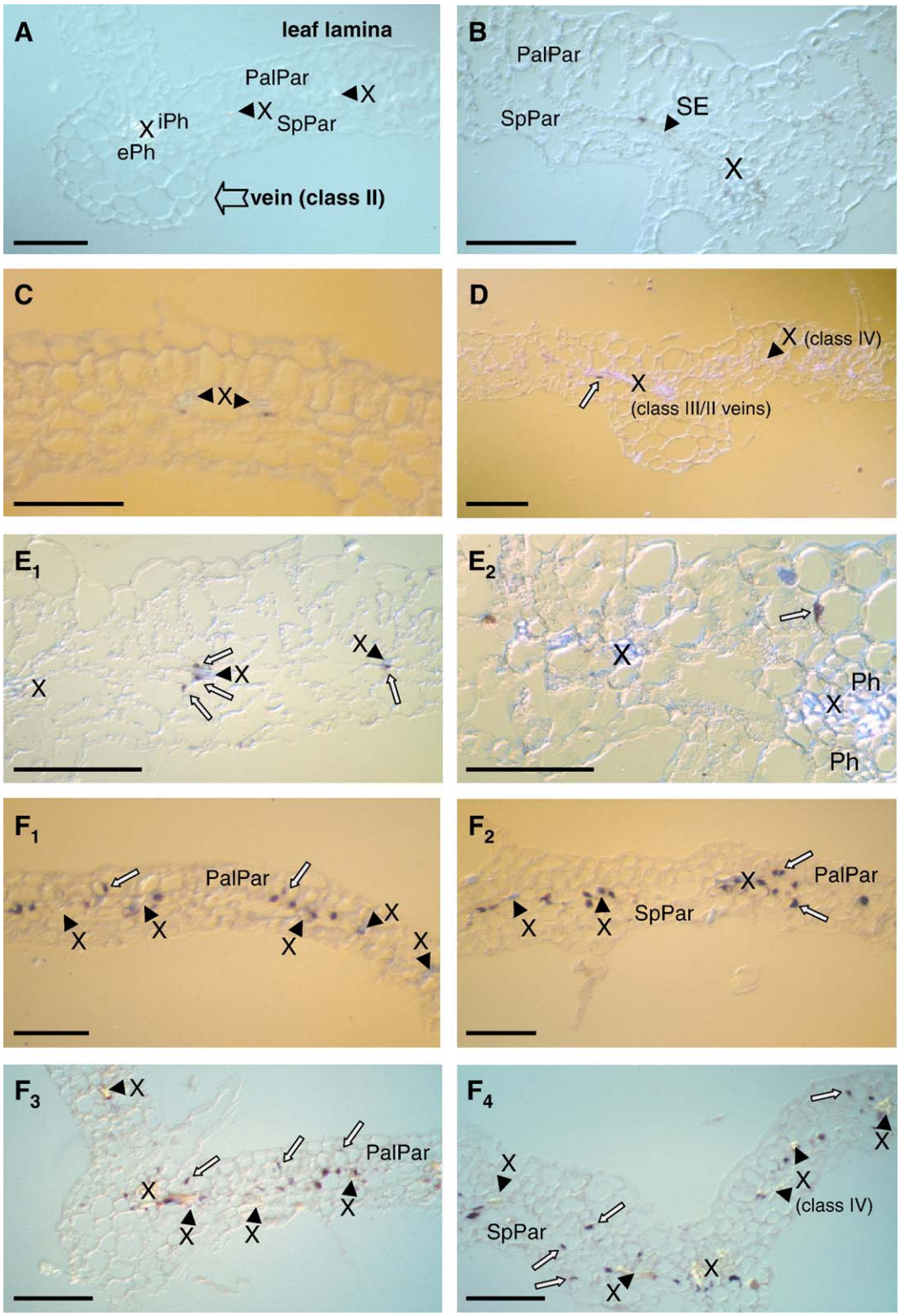

Fig. 5. *In situ* detection of AbMV DNA (dark stain) in leaf sections of *N. benthamiana* plants embedded 40 to 45 dpai. (A) Mock-inoculated control. (B, C) AbMV-infected plants (specimens from B: mature leaf, at minimum 2 cm in length, or C: young leaf). (D–F) Sections from AbMV/Fny-CMV doubly infected plants; (D) Young green, slightly wrinkled leaf showing milder symptoms than others. AbMV remains confined to the phloem (arrow). ($E_{1,2}$) Fully expanded leaves exhibiting symptoms of intermediate intensity. Arrows point at AbMV-containing nuclei adjacent to each other (E_1), or at some AbMV-infected parenchyma cells obviously outside the phloem (E_2). (F_{1-4}) Heavily affected, chlorotic young leaves: Signals on AbMV-infected nuclei in nonphloem tissues are indicated by arrows. Differential Contrast Microscopy (DIC); X: xylem, (e/i) ph: (external/internal) phloem, PalPar: palisade parenchyma, SpPar: spongy parenchyma (representative marking). Scale bars represent 50 μm.

Table 1
Numbers of AbMV-infected cells counted in sections from different leaf types and of different co-infection status as indicated

Plant	Infection status	Leaf type[a]	Median number: infected cells	Minimum number: infected cells	Maximum number: infected cells	Number of slides analyzed	Number of sections analyzed
N. benthamiana	AbMV*†	y*,†	4.0*†	1.0	8.7	21	208
	AbMV	m	2.5	2.0	3.3	6	56
	AbMV/Le-CMV*	y*	6.9*	2.2	15.0	13	153
	AbMV/Le-CMV	m	3.7	1.5	14.5	7	86
	AbMV/Fny-CMV†	y/ch†	19.0†	2.5	33.8	7	95
	AbMV/Fny-CMV	y/g	3.1	1.1	6.3	6	74
	AbMV/Fny-CMV	m/ch	3.1	2.7	3.5	2	23
	AbMV/Fny-CMV	m/g	2.1	1.3	4.0	4	44
L. esculentum	AbMV	y	19.1	6.8	35.6	8	99
	AbMV	m	11.1	0.8	26.9	6	69
	AbMV/Le-CMV	y	8.6	3.1	40.1	10	127
	AbMV/Le-CMV	m	4.6	1.7	5.9	8	82
	AbMV/Fny-CMV	y	16.5	12.6	39.3	9	100
	AbMV/Fny-CMV	m	19.3	1.5	34.5	4	51

Section length (i.e. specimen width of embedded leaf lamina) was measured for each slide, the mean signal number per section determined for series of usually 6 to 15 consecutive sections, then normalized for a standard section length of 2.5 mm and subjected to statistical analysis using the nonparametric Mann–Whitney rank sum test on median values. For *N. benthamiana*, 528 sections from 34 leaves on 66 slides, and for tomato, 739 sections from 24 leaflets on 45 slides were analyzed (12,483 hybridization signals, typically two slides per specimen). "Minimum/maximum numbers of infected cells" refer to minimum or maximum mean values calculated for the standard width of the specimen, "median numbers" were determined from all individual mean values of slides representing same infection status and leaf type. *,†Statistically significant difference for $\alpha < 0.05$ as validated by Mann–Whitney Rank Sum Test, $P_{error} = 0.0119$ (*) or 0.0296 (†). [a]Leaf type: "y": young, "m": mature/fully developed, "ch": chlorotic/yellow, "g": green.

total number of infected cells was extraordinary low, about one tenth of that of *N. benthamiana* and 1/20th to 1/50th of that of tomato (Table 1). In addition, the intensity of hybridization signals, which were detected only in veins, was equal to that in nontransgenic tobacco (Fig. 8). By contrast, *N. tabacum* line C2bx6b5 revealed a remarkably increased number of AbMV-containing cells in most leaves analyzed: In young lamina, the median number of virus-specific signals was 17 times that of the nontransgenic siblings (* in Table 2), which strongly exceeds the two- to fivefold enhancement determined for mixed infections in *N. benthamiana*. For mature leaves, a sevenfold increase was observed in relation to comparable leaves of all control groups. The median number of AbMV-accumulating nuclei in leaves from 2b-expressing individuals in this plant line did not differ notably between tissues embedded before and after the change in light and temperature. Altogether, 7 out of the 11 specimens from distinct CMV 2b-expressing plants (Table 2) exhibited markedly raised numbers of AbMV-containing cells, 5 of them being sampled before, and 2 after the change in cultivation conditions. These findings again established that the begomoviral DNA content in plant tissues did not necessarily reflect the number of infected nuclei.

Despite of the prominently enhanced portion of infected cells, any AbMV-infected nonvascular cell was discovered in none of more than 400 sections analyzed (Fig. 8). Begomoviral DNA was detected in nuclei of companion and phloem parenchyma cells in the external and internal phloem tissues, the latter of which in some cases were located close to adaxial (upper) leaf surface in minor veins (as demonstrated in panel A_1) in this plant species. Notwithstanding, all signals could be clearly attributed to vascular cells, indicating that the presence of CMV 2b protein did not alleviate the phloem limitation of AbMV.

Discussion

Our systematic analysis on the combination of ssDNA and plus-strand ssRNA viruses in common host plants resulted in a number of striking findings. The bipartite begomovirus AbMV, which up to now had been localized exclusively to the phloem of different host plants and exhibits a rather limited pathogenicity by itself, has been shown to contribute to a strong synergism of symptoms with CMV. Quantification of plant height or biomass revealed that mixed infections of the DNA virus with either Fny- or Le-CMV resulted not only in additive, but also in synergistic symptom enhancement in three host species. Blot and ELISA analyses showed that AbMV accumulated to evidently increased titers in the presence of CMV, the amount of which remained unchanged. Concomitantly, an increased number of *N. benthamiana* nuclei were shown by *in situ* hybridization to contain geminiviral DNA. Moreover, CMV potentiated spread and altered the tissue specificity of AbMV, which in mixed infections was able to escape from the phloem, and to invade palisade parenchyma cells of the mesophyll. Whereas tobacco expressing functional CMV 3a movement protein did not support any of the effects observed, the presence of CMV 2b silencing suppressor protein led to enhanced AbMV titers and strongly increased numbers of infected vascular cells in the *N. tabacum* cv. Samsun nn x Paraguay PBD6 line C2bx6b5 (Guo and Ding, 2002).

Symptom phenotype and tissue specificity of geminiviruses may be influenced by a range of completely different viral functionalities, and depend on the interplay with the particular host species. Presumably, duration and effectiveness of virus adaptation to the host strongly influence those features. Interesting host-dependent differences have been found for *Euphorbia mosaic virus* (Kim and Lee, 1992), *Sri Lankan*

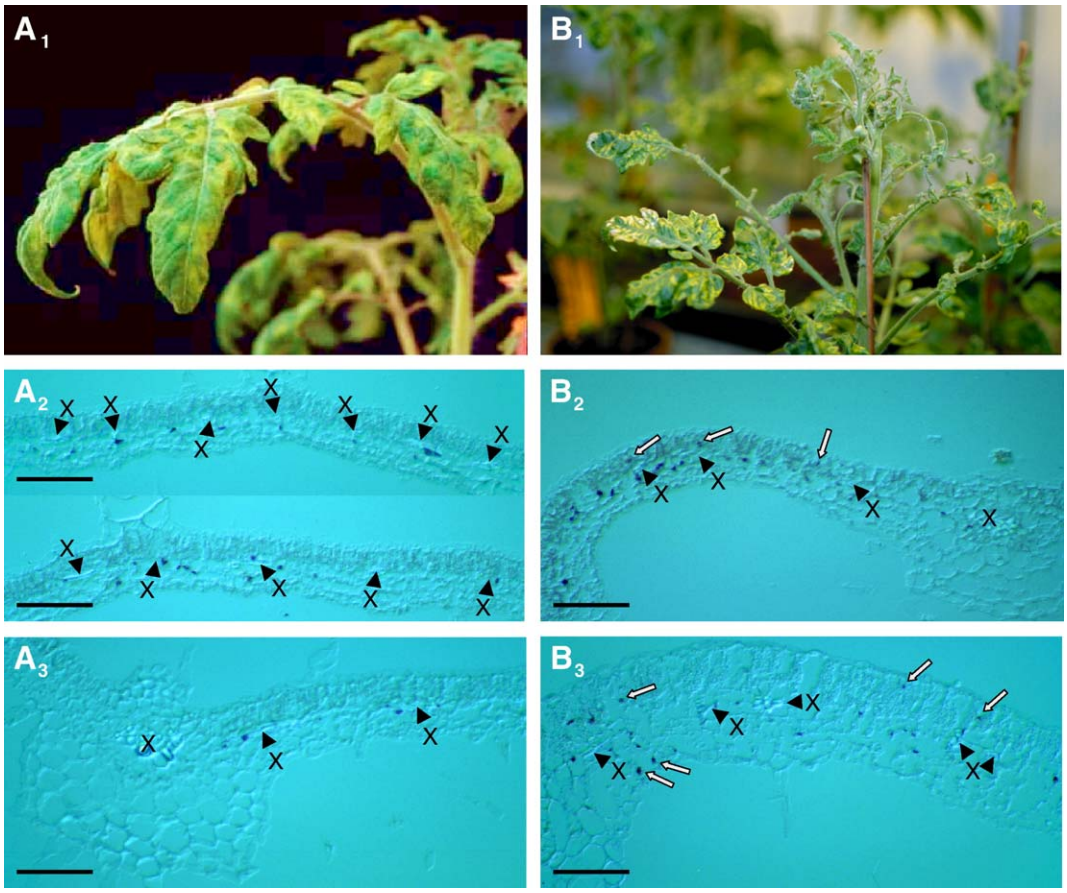

Fig. 6. Singly AbMV (A) and AbMV/CMV doubly infected (B) tomato plants: symptoms and tissue tropism of AbMV. Column A: Symptoms (A_1), *in situ* detection of AbMV DNA in phloem nuclei (A_2: representative sections of young, A_3: of adult leaves). Column B: Symptoms on AbMV/Le-CMV mixed-infected tomato (B_1) and the respective tissue distribution of AbMV DNA in a young leaflet (B_2: leaf lamina not completely reduced, therefore low to intermediate symptom intensity). (B_3) AbMV/Fny-CMV mixed infected tomato leaflet from mature pinnate leaf of intermediate symptom intensity. Arrows in panels B_2 and B_3 point at nuclei of palisade or spongy parenchyma cells (as verified by the absence of adjacent vascular elements in consecutive sections). X tags representative xylem elements (cross- or longitudinally sectioned). Note that tomato nuclei are smaller or more condensed than those of *N. benthamiana*, which makes photographic documentation of the hybridization signals difficult. Specimens were embedded at 28 dpai, phenotype photographs taken at 35 dpai. DIC microscopy, scale bars represent 50 μm.

cassava mosaic virus (Saunders et al., 2002), *Bean dwarf mosaic virus*, *Tomato mottle virus* (Hou et al., 1998; Wang et al., 1999), *Bean golden mosaic virus* and *Tomato golden mosaic virus* (Morra and Petty, 2000). The experiments have revealed that in certain bipartite begomoviruses both genome components, while in others only one, determine(s) host-specific tissue tropism (Hou et al., 1998). For individual viruses, phloem limitation was shown to correlate with a noncoding DNA B sequence in combination with either certain regulatory DNA A (AC2), or alternatively specific DNA B transport proteins (Morra and Petty, 2000). It could arise from replication-associated proteins (encoded by ORFs *[A]C1*) failing to interact with plant cell-cycle regulators like pRBR (Kong et al., 2000; McGivern et al., 2005). (A)C3 (Replication Enhancer, REn) proteins involved in the replication-cell-cycle connection also contribute to tissue tropism (Settlage et al., 2005), and geminivirus accumulation and symptoms may depend on the posttranscriptional gene silencing suppressors encoded by

Fig. 7. Southern blot analysis revealing relative levels of AbMV DNA in CMV 2b-transgenic, and the respective control plants in *N. tabacum* cv. Samsun nn line C2b (left column, A to C) and *N. tabacum* cv. Samsun nn x cv. Paraguay PBD6 line C2bx6b5 (right column, D to F) in leaves of different ages at different dates post agroinfection; representative assays defining the genetic background (G, H), and phenotype of typical plants (I; A, D, G–I: prior, B, C, E, F: after transfer to high light/temperature conditions). Lanes represent individual plants: 1, 2, 20, 21: mock-inoculated, 3–9 and 22–26: nontransgenic control plants in the same progeny as the corresponding transgenic plants 10–14 and 27–34. 15–19: *N. tabacum* cv. Samsun nn nontransformed plants from the same origin as C2b-transformed plants. (A, D) Samples from young leaves harvested 63 dpai, (B, C, E, F) samples from 95 dpai (B, E: young, C, F: mature leaves, respectively). Samples containing equal amounts of plant genomic DNA were separated on 0.8% agarose gels containing EtBr, blotted and hybridized as described in the text. S_{1-3}: Standard, linear AbMV DNA A (1/10/100 pg). AbMV DNA forms indicated by arrows as described for Fig. 2. (G) Kanamycin resistance assay on MS callus induction plates showing representative samples; left: kanamycin-sensitive, right: -resistant tissues. The assay started at 50 dpai (photo taken after 25 days of leaf explant cultivation; refer to Materials and methods). (H) GUS activity assay with representative leaf explants from plants in line C2bx6b5, showing release of GUS silencing in both veins and lamina in 2b-expressing plants (right) as compared to silenced GUS expression in veins in 2b-nontransgenic ones (left; GUS staining for 16 h at 37 °C, as described in Materials and methods). (I) Plants no. 24 (nontransgenic) and 29 (2b-expressing) in line C2bx6b5, at 51 dpai.

ORFs *AC4* or *AC2* (for reviews on geminiviral silencing suppressors, refer to Bisaro, 2006; Vanitharani et al., 2005).

For AbMV, however, as for other phenotypically similar important tomato begomoviruses (Morilla et al., 2004), the molecular basis for the restricted tissue infiltration competence has not been delimited yet. Possibly, it is based on more than one viral element since absence from nonphloem cells in systemically invaded leaves, in combination with moderate disease

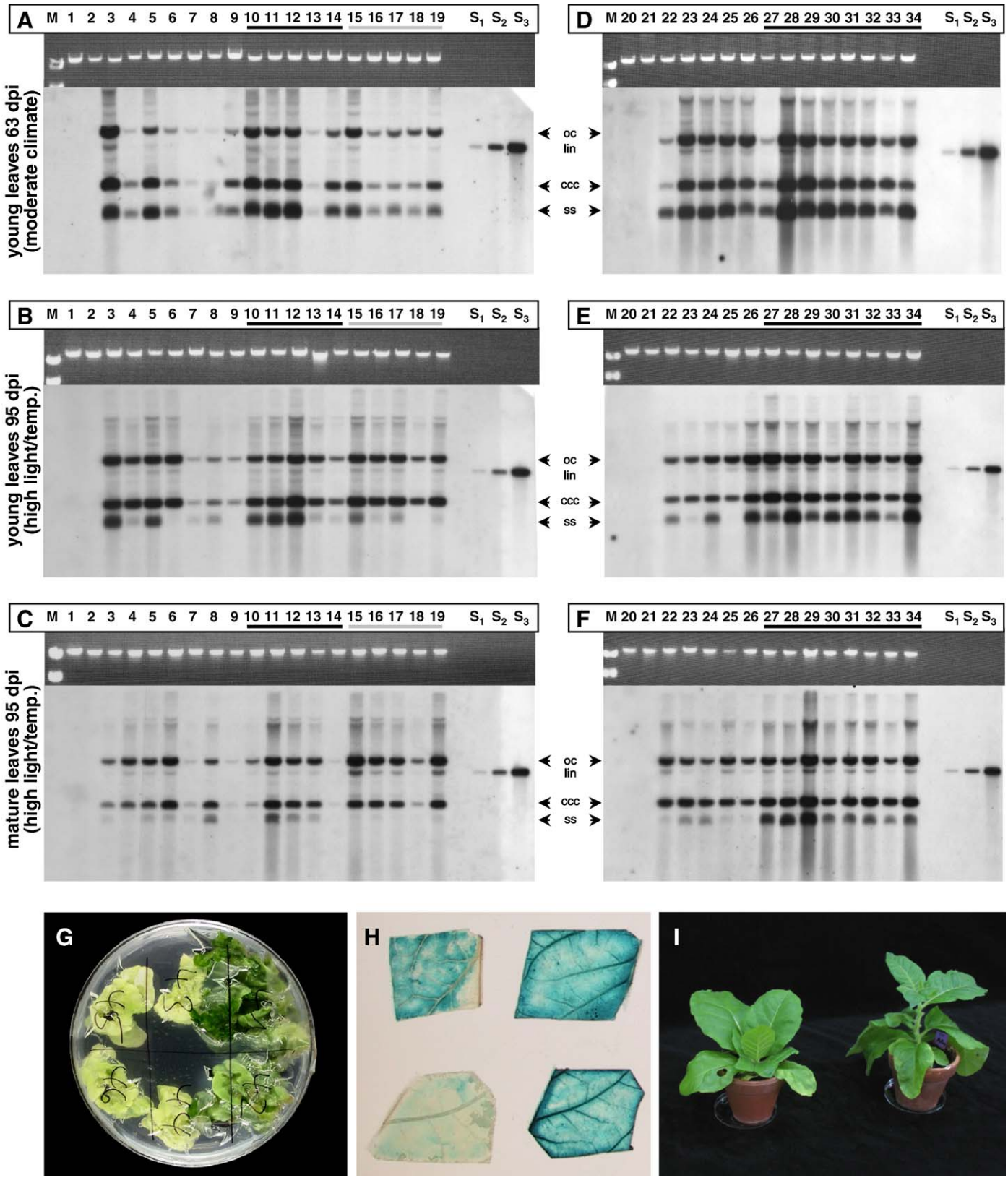

Table 2
Numbers of infected cells counted in sections from different leaf types of AbMV-infected CMV 2b protein-transgenic and control *N. tabacum* plant lines as indicated

Plant line[a]	Expression of CMV 2b protein	Leaf type[b]	Median number: infected cells	Minimum number: infected cells	Maximum number: infected cells	Number of specimens/ slides analyzed	Number of sections analyzed
N.t. cv. S. nn (wt)	Negative	y	0.2	0.0	4.3	4/6	123
	Negative	m[c]	0.4[†2]	0.0	3.3	4/4	97
N.t. cv. S nn C2b x wt	Negative	y	0.8	0.2	2.5	6/10	181
	Negative[d]	m[d]	0.4[d]	0.4	0.4	1/2	15
	Positive	y	0.4	0.2	5.5	5/8	140
	Positive	m	1.2	0.0	1.9	3/6	48
N.t. cv. S. nn x Par. C2b x 6b5	Negative	y*[†]	0.5*[†1]	0.0	2.1	4/8	98
	Positive	y*	8.5*	0.3	16.7	7/11	291
	Positive	m[c]	3.4[†1/2,d]	0.4	6.3	4/7	124

Normalization of signal numbers and nonparametric Mann–Whitney rank sum test on median values was done as explained in Table 1. 1117 sections from 38 leaves on 62 slides were analyzed with repetitions in three independent ISH experiments (4262 hybridization signals were counted in sections from individual specimens on two to four slides each; most slides combined sections from different specimen types adjacent to each other). "Minimum/maximum numbers of infected cells" refer to the mean values calculated for the standard width of the specimen, "median numbers" were determined from all individual mean values of slides representing the same genetic background and leaf type. Statistically significant difference for $\alpha < 0.05$ as validated by Mann–Whitney Rank Sum Test, $P_{error} = 0.003$ (*), or 0.018 ([†1])/0.017 ([†2]). [a]Plant lines: *N.t.* cv. S. (*N. tabacum* cv. Samsun) nn, or nn x Paraguay PBD6. [b]Leaf type: "y": young, "m": mature/fully developed. [c]Since mature leaves from non-expressing C2bx6b5 plants were not included in the analyses, median AbMV-specific signal numbers for 2b-expressing mature leaves were compared to young homologous, or mature wild type leaves, respectively, the median values of all of which do not differ significantly. [d]Power of putative test insufficient due to low number of sections/specimens.

symptoms, seems to be a very stable and fundamental property of AbMV. This not only occurs in its ornamental host *Abutilon sellovianum* REGEL as a result of mutual long-time evolution (Wege et al., 2000), but also in malvaceous weeds, the laboratory host *N. benthamiana* (Wege et al., 2001), and, as shown in this study, in crops belonging to the *Solanaceae*. This constant behavior was a reliable prerequisite for detection of the positive viral interactions with respect to symptoms, virus accumulation and tissue distribution. On the other hand, cycling of CMV symptoms, most prominent in tomato, resulted in a variable but reproducible situation that required long-time experiments and standardized sampling at different developmental stages. The cycling phenomenon has been attributed to changing levels and subcellular tropisms of CMV 3a MP. It is thought to reflect stages of excessive virus movement (Gal-On et al., 1995; Gal-On et al., 1996). Interestingly, the more heavily cycling Le-CMV-infected tomatoes developed a stronger symptom synergism with AbMV than did the Fny-CMV. This suggested possible interactions between the transport machineries of the viruses.

The 3a MP of CMV harbors intrinsic features responsible for phloem export under certain conditions (Itaya et al., 2002), and plants transgenic for the *3a* MP ORF have been successful in supporting movement of unrelated viruses (Kaplan et al., 1995). The protein has a capacity for plasmodesmatal gating (Ding et al., 1995; Vaquero et al., 1994) and for ssDNA binding (Li and Palukaitis, 1996). Notwithstanding, expression of CMV 3a MP in tobacco neither influenced symptoms, nor supported phloem release of AbMV. Although we cannot rule out a contribution of the CMV MP to mesophyll infiltration by AbMV in the presence of other CMV-associated factors during mixed infections, our data may also indicate that transport complementation is not involved in the "enhanced spread" phenotype.

Besides movement proteins, plant viral RNA silencing suppressors are further key components in long-distance and cell-to-cell spread, influencing also symptoms and the overall accumulation of viruses (for a recent overview, refer e.g. to Qu and Morris, 2005). Well-known examples are the potyviral helper component proteinases (HC-Pro) which were shown to amplify replication and pathogenicity of several unrelated viruses (for review, refer to Palukaitis and MacFarlane, 2006). *Tobacco etch virus* (TEV) HC-Pro also accounted for a strongly enhanced tissue infiltration of the otherwise largely phloem-limited *Potato leafroll virus* (PLRV) when expressed in transgenic tobacco, increasing the number of PLRV-invaded mesophyll cells by a factor of 450 in comparison to non-transgenic plants (Barker et al., 2001). So obviously, tissue-specific antiviral defense responses contributed to the restriction of PLRV to the veins, an explanation which is supported by other recent findings on tissue-specific silencing in plants (Andika et al., 2005; Tuteja et al., 2004). In analogy to the findings with HC-Pro, the cucumoviral 2b PTGS suppressor exerted a similar influence on PLRV, mediating its spread into mesophyll parenchyma (Ryabov et al., 2001). The effect, however, was additionally dependent on the presence of an umbraviral MP.

Since its transiently expressed movement proteins were shown to function in tissues other than phloem (Zhang et al., 2001), it is likely that AbMV is indeed mainly impaired in a putative mesophyll PTGS suppression competence and thus confined to the phloem as the result of an adaptation to *Abutilon* plants combating the virus. A gene silencing-suppressing ORF for AbMV has not yet been determined. So we decided to test if CMV 2b-expressing plants were able to mimic all the effects we had observed in the mixed infections, as they were symptom and DNA accumulation enhancement, and export from the vascular tissues. We failed, however, to detect any differences in symptomatology between AbMV-infected transgenic and the nontransgenic sibling plants since individual plants in both 2b-transformed lines were growing very heterogeneously. This might have masked any minor increase in AbMV-caused stem stunting in the 2b-expressing plants, the symptom which was the main indication for synergism in the mixed infections in tobacco.

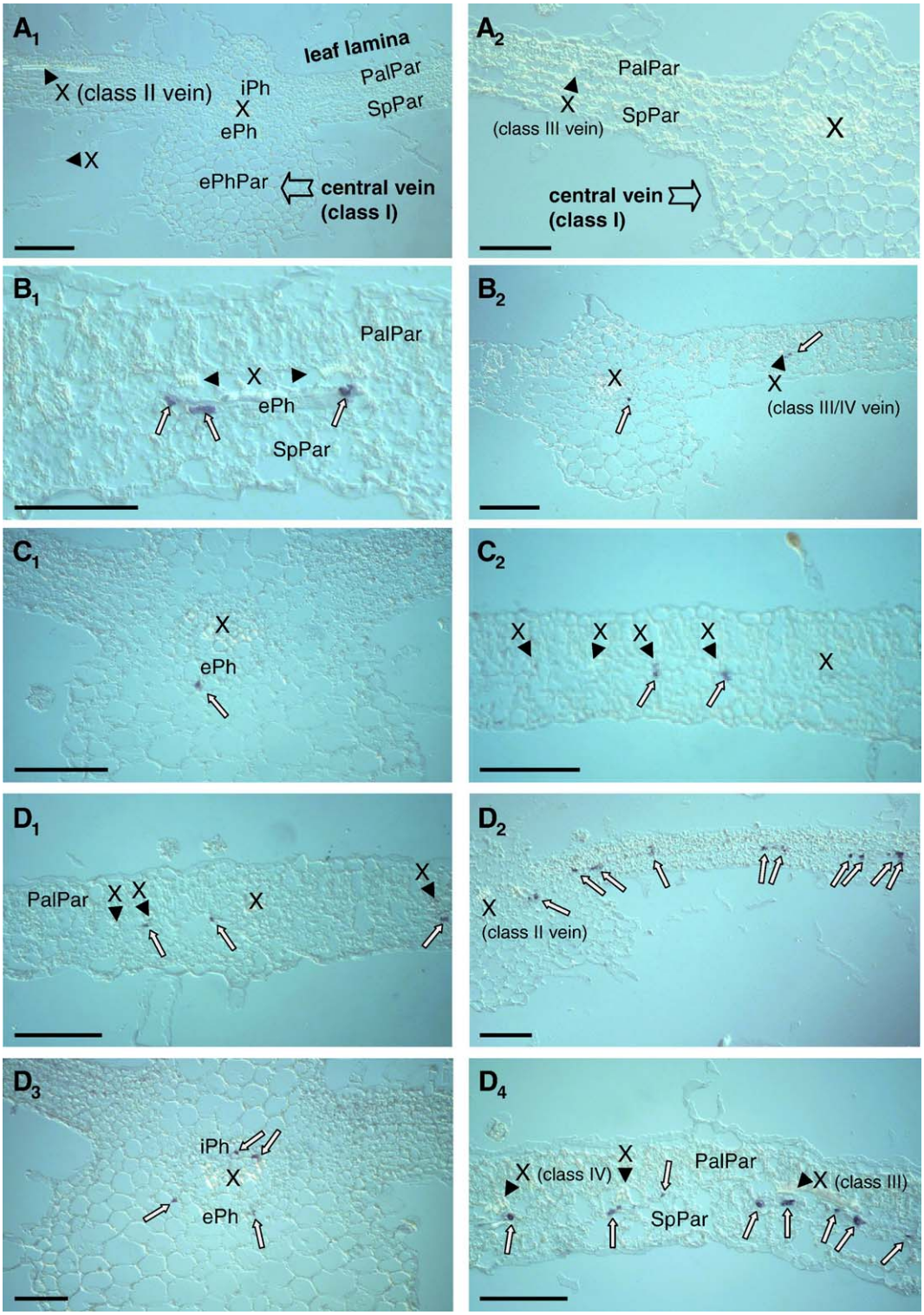

Fig. 8. *In situ* detection of AbMV DNA (dark stain) in leaf sections of *N. tabacum* plants of different genetic background, C_2, D_2 and D_4 expressing CMV 2b protein. (A) Mock-inoculated controls, young leaves (A_1: nontransformed *N. tabacum* cv. Samsun nn, embedded at 71 dpai; A_2: nontransgenic plant from line C2bx6b5, 42 dpai). (B) Nontransformed *N. tabacum* cv. Samsun nn, AbMV-infected (B_1: young leaf, 71 dpai; B_2: mature leaf, 42 dpai). (C) Line C2b, young leaves, C_1: nontransgenic (71 dpai), C_2: expressing 2b protein (99 dpai). (D) Line C2bx6b5, D_1/D_3: nontransgenic plants, young leaves (71 dpai, D_1: leaf lamina, D_3: central class I vein); D_2/D_4: young leaves expressing 2b protein (99 dpai). Arrows point at AbMV DNA-specific signals. Arrowheads labeled "X" indicate xylem elements. Differential Contrast Microscopy (DIC); (e/i) Ph: (external/internal) phloem, PhPar: phloem parenchyma, PalPar: palisade parenchyma, SpPar: spongy parenchyma (representative marking). Scale bars represent 50 μm.

In every synergistically diseased doubly infected plant analyzed, AbMV DNA titers were clearly elevated. However, a different behavior of the three solanaceous species was noticeable: Virus DNA accumulation was enhanced either from an early developmental stage on in every single leaf of *N. benthamiana* by a factor of about five to fifteen, or starting at different stages of leaflet growth in tomato, reaching roughly two- to tenfold AbMV titers. At least in *N. benthamiana*, increased amounts of begomovirus DNA were detectable over prolonged periods of time, also in fully developed leaf tissues. In contrast, in *N. tabacum* AbMV DNA levels were markedly increased only transiently in young leaves. In *N. benthamiana*, the unusually high accumulation of AbMV DNA was associated with a significantly raised number of infected nuclei, as counted in tissue specimens following *in situ* detection of AbMV DNA. In tomato leaf sections, no such observation could be identified, which may be due to sampling errors. On the other hand, the relative intensity of nucleus-specific signals developing upon *in situ* detection of AbMV DNA in doubly infected specimens seemed to be unusually high in comparison to that in singly infected tissues. Unfortunately, the detection procedure using nonradioactive probes and nonfluorescing substrates did not allow for quantification of single signals, so this conclusion is based only on comparative microscopic evaluation of specimens. Both effects, enhancement of AbMV DNA titers and numbers of invaded nuclei, were unequivocally reproduced by the CMV 2b-expressing plants in line C2bx6b5. To our surprise and different from the mixed infections in tobacco, increased levels of viral DNA by roughly a factor of 5 to 10 persisted in mature leaves. Since the number of infected nuclei was enhanced by 7 in the old, and by 17 in the young leaves, the gain in AbMV titer was mainly achieved by infection of additional susceptible cells in the transgenic plants, and not dominantly by accumulation of elevated amounts of AbMV DNA inside nuclei. This differed from our findings for doubly infected *N. benthamiana* and tomato, in which both mechanisms were likely to act in parallel.

Therefore, although CMV 2b was shown to support multiplication of heterologous RNA viruses (Liu et al., 2002), we only could prove its effect on the number of AbMV-invaded cells in tobacco, although geminiviral ssDNA replication indeed seems to be restrained by RNA-initiated feedback inactivation of DNA templates (Matzke and Birchler, 2005) and thus might benefit from the presence of a PTGS suppressor. Furthermore, we did not obtain any evidence that 2b protein by itself can assist AbMV in exiting the phloem. This resembles findings on PLRV in which *Potato virus A* (PVA) HC-Pro was able to increase the luteoviral titer in *N. benthamiana*, but did not alter its confinement to vascular cells, which was in contrast to mixed infections when additional potyviral moieties were present (Savenkov and Valkonen, 2001). Hence it is probable that CMV 2b protein is necessary, but not sufficient to alleviate the phloem limitation of AbMV; further CMV protein(s) – which may include the 3a MP – could contribute. In a comparable study on *Potato virus Y* (PVY), which was confined to the external phloem of tobacco stems in single infections, a CMV 2b-dependent egress of the potyvirus into nonphloem tissues was observed (Ryang et al., 2004), as proved by a mutant virus lacking a translatable ORF 2b which failed to support PVY. The use of a virus deficient in the PTGS suppressor, however, did not allow for a decision if 2b protein had been sufficient to function in phloem export or not; furthermore, it might be complicating experiments due to altered movement characteristics of the resulting virus (Soards et al., 2002), why we decided to use 2b-expressing plants in order to test for a role of the PTGS suppressor in the synergism.

Although, one of the CMV 2b-transgenic plant lines failed to support AbMV. This may be explained either by a lower level of protein, which was not analyzed in our study, or by the different genetic background of *N. tabacum* cv. Samsun nn x *N. tabacum* cv. Paraguay PBD6. Since both lines were transgenic for the 2b protein of Q-CMV belonging to the milder subgroup II of cucumoviruses, it cannot be excluded that in a plant line expressing limited amounts of the protein any measurable effect will stay away. It has been established that 2b proteins of subgroup I and II are similar in their mode of action, but mediate distinct levels of virulence (Shi et al., 2002). This might also explain the failure of the C2bx6b5 plants to mediate egress of AbMV into cells outside the phloem. Furthermore, subgroup I and II 2b proteins seem to slightly differ in their activity patterns inside the plant cormus (Bucher et al., 2003). This might contribute to our findings showing that, in 2b-transgenic tobacco, AbMV replication was not only clearly enhanced in young, but also in mature leaves, which differed from the natural co-infection. It is more likely, however, that this discrepancy reflected the constant presence of 2b protein, which was not given on the background of a cycling systemic CMV infection.

After a rise of light and temperature, CMV 2b-expressing plants accumulated further increased levels of AbMV. This might indicate a positive correlation between the begomovirus-promoting PTGS suppression activity of CMV 2b and elevated temperature, although the effect was not separated from the influence of light intensity. The finding contrasts with observations for other viruses, which in most cases were shown to overcome the antiviral silencing defense to a higher extent at low temperature (Qu and Morris, 2005), but is in analogy with a report on systemic CMV infection in a plant in the family *Aizoaceae*, which was only established at increased temperatures (Kobori et al., 2003).

Both, CMV subgroup I and subgroup II 2b proteins depend on nuclear localization for mediating PTGS suppression (Lucy et al., 2000; Wang et al., 2004), like it has been established for begomoviral silencing suppressors, too (Bisaro, 2006, and references herein). For the 2b protein, it was shown that upon its activity inside the nucleus, it prevents the systemic spread of an antiviral silencing signal to differentiating tissues (Roth et al., 2004, and references therein). It may be speculated that 2b either acts on the same pathways like PTGS suppressors of nonphloem-limited begomoviruses, complementing the deficiencies, or that it addresses alternative pathways gating AbMV into additional cells of the vascular tissues. This may occur in conjunction with a transactivation of beneficial host genes such as encoding cell-cycle promoting factors in order to regenerate tissues (Trinks et al., 2005; Wang et al., 2003, 2005).

It is also possible that secondary effects of CMV exert an additional indirect influence on AbMV translocation, promoting escape from the phloem. Plant transport fluxes, namely active phloem unloading mechanisms, may be affected by a large variety of signaling events (Oparka and Santa Cruz, 2000). This includes responses to altered carbohydrate levels (Koch, 1996), which in doubly infected plants may be caused by the cucumoviral or by synergistic symptoms. Resulting quantitative or mechanistic changes in phloem unloading might, concomitantly, benefit AbMV. ELISA analyses showed that, in the mixed infections, CMV levels were not significantly altered. In most other reports on plant viral synergism involving CMV, the cucumovirus increases in accumulation.

With respect to the interaction between DNA and RNA viruses within plants, our findings are more surprising than those described previously. To our knowledge, the only two studies available on double-infections with dsDNA caulimoviruses and RNA viruses report either negative interference with a potyvirus (Kamei et al., 1969), or – despite symptom enhancement – mutually unchanged virus levels in co-infection with a tobamovirus (Hii et al., 2002). A previous electron microscopic investigation, however, indicated a further positive ssDNA-RNA virus interaction at the cellular level: *Bean golden mosaic virus* was shown to enter mesophyll parenchyma only in the presence of a tobamovirus in common bean (Carr and Kim, 1983). No effects on symptomatology or virus DNA titers were described. Thus, the present study substantiates that previous suggestion based solely on microscopic analysis of double-infected plants and extends it with novel observations on ssDNA-RNA virus interactions at the molecular level. Our conclusions suggest that this type of mixed infections may have a potentially ecological and economic relevance. Strongly increased geminivirus titers may enhance insect vector transmission efficiencies as has been shown previously for other viruses (Rochow, 1972). An extended spatial tissue distribution including the mesophyll parenchyma domain might also raise the effectiveness of mechanical virus dissemination, as was demonstrated for phloem-limited luteoviruses invading mesophyll cells in co-infection with an umbravirus (Mayo et al., 2000; Ryabov et al., 2001). High numbers of genome molecules in increased numbers of cells could elevate the frequency of homologous recombination events and thus built a positive evolutionary driving force. Concomitantly, epidemiological risks due to the use of plants transgenic for geminivirus-derived sequences have to be re-evaluated (Jeske, 2002; Tepfer, 2002). And finally, if mixtures of two or more geminiviruses replicate in CMV-co-infected tissues under conditions of selection, this may increase the probability of new epidemics. Selection could be due to newly imported crop cultivars or to infestation by viruses new to the environment.

Materials and methods

Plants and viruses

N. benthamiana DOMIN, *N. tabacum* cv. Turkish Samsun nn and NN (wild type and Fny-CMV protein 3a-expressing plants, line 3a-3, as described by Kaplan et al., 1995, the latter kindly provided by Prof. Dr. Peter Palukaitis, SCRI, Dundee, Scotland) and *Lycopersicon esculentum* L. cv. "Moneymaker" were cultivated in an insect-free S2 containment greenhouse with supplementary lighting (100 kW/h), with a 16 h photoperiod at 25 °C and a night-time reduction to 20 °C at 60% rel. humidity. *Nicotiana tabacum* cv. Samsun nn, raised from nontransgenic seeds of different origin, and a corresponding Q-CMV-2b (Ding et al., 1994) transgenic hemizygous line (progeny of male-sterile parental line C2b14, described in Ji and Ding (2001)) as well as a hemizygous offspring line derived from a crossing of line C2b14 with GUS-transformed *N. tabacum* cv. Paraguay PBD6 (Elmayan and Vaucheret, 1996), named "C2bx6b5" (Guo and Ding, 2002; all three lines by courtesy of Prof. Dr. S.-W. Ding, University of California, Riverside, U.S.A.), were grown in a controlled climate chamber for about four months (16 hrs photoperiod; day/night 23/20 °C) and then transferred to the greenhouse with supplementary lighting, with a 16 h photoperiod at 30 °C and a night-time reduction to 18 °C.

AbMV was delivered to plant seedlings via agroinfection (Klinkenberg et al., 1989) by use of *Agrobacterium tumefaciens* LBA 4404 (Hoekema et al., 1983), clones AbA (AbMV DNA A) and AbB (AbMV DNA B; Frischmuth et al., 1993). Virion preparations of two CMV subgroup IA strains were kindly donated by Dr. K.-H. Hellwald, formerly Stuttgart-Hohenheim. Fny-CMV originating from New York (Roossinck and Palukaitis, 1990) had been isolated from *N. tabacum* plants inoculated with *in vitro* generated transcripts of cloned Fny-CMV (Rizzo and Palukaitis, 1988, 1989, 1990; Roossinck and Palukaitis, 1990). Le-CMV from Japan (Takeshita et al., 2004a; Tomaru and Udagawa, 1967; Zaitlin et al., 1994) originated from *N. tabacum* inoculated with legume-infective virions originally selected and propagated in laboratory hosts (clones and primary inocula were kindly provided by Prof. Dr. P. Palukaitis; for review, see Palukaitis et al., 1992). Virion preparations (50 µg/ml each, in 0.1 M sodium phosphate buffer pH 7.1) of Fny- or Le-CMV were used for mechanical inoculation of two individual, Carborundum (320 mesh)-dusted leaves per test plant (10 µl inoculum per leaf; Rawlins and Tompkins, 1936) either directly prior to, or 6 to 12 days post agroinoculation. Control plants were similarly treated with buffer in the case of CMV, or clone AbB in the case of AbMV.

Analyses on plant genetic backgrounds and expression status of transgenes

Transgenic and transgene expression status of the plants in both hemizygous CMV 2b-transformed tobacco lines was analyzed by means of different techniques, all of them utilized for every single plant in the experiments (with the exception of GUS assays, which were only conduced on plant line C2bx6b5 and a number of control plants from different lines). PCR was carried out on total nucleic acid preparations obtained as specified below. For RT-PCR, verifying the transcription of genome-integrated RNA 4A,

total plant RNA was extracted from single young leaves (10 to 15 mm in length, ground in liquid nitrogen) by use of TRIZOL reagent (Invitrogen), following the manufacturer's protocol. Absence of contaminating RNA was verified by test reactions carried out with the RT-PCR system (as specified below) after heat inactivation of the reverse transcriptase contained in the kit. Both PCR and RT-PCR used a 20mer-primer pair with the upstream primer binding to positions 28 to 47, and the downstream primer to the complementary sequence of positions 342 to 323 in Q-CMV RNA 4A (GenBank accession no. Z21863), applying standard (RT-)PCR programs (annealing temperatures 58 °C). For PCR, Qiagen Taq DNA polymerase (Qiagen, Hilden, Germany) was used on 10 µg of total plant DNA with the reagents supplied, according to the enclosed instructions. RT-PCR was done on 1/6 of the total RNA extracted from a small leaf with downscaled (half-volume) reactions of the "Titan One Tube RT-PCR System" (Roche, Grenzach, Germany), following the recommended protocols. GUS and kanamycin resistance assays were carried out with tobacco leaves 3 to 7 cm in length. In GUS staining tests, the method of Anandalakshmi et al. (1998) was followed, using 4 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) staining solution supplemented with 0.1% Triton X-100 at 37 °C for 16 h. The kanamycin resistance status of leaf tissues was determined on callus induction agar plates composed of salts and organic compounds according to Murashige and Skoog (1962), supplemented with 1 mg/l 6-benzylaminopurine, 0.1 mg/l 1-naphthaleneacetic acid, 30 g/l sucrose and 75 mg/l kanamycin. Sterilized leaves were rinsed in water, shortly dried on filter paper, cut along the edges and several times cross the central vein and placed topside-down on the agar plates which were sealed with Parafilm and cultivated in inverted position at 24 °C and permanent light for 1 week. After transfer of the leaf explants to fresh MS-kanamycin plates, cultivation continued for at least two further weeks until bleaching of kanamycin-sensitive tissues was obvious.

Symptom analyses

Symptom analyses were carried out starting from 16 dpai up to 63 dpai, in the case of 2b-transformed plant lines up to 99 dpai. Photographs were taken on Kodak Ektachrome 50 film with an Olympus OM4-Ti mirror reflex camera or with a Canon PowerShot G5 digital camera. Stem height from soil surface up to the apical leaf bud and dry weight of individual plants' above ground biomass were determined (drying conditions: 60 °C for 4 weeks). Statistical data evaluation was carried out using SigmaStat software (for Windows, version 1.0, 1992–94 Jandel corporation, 1993 MicroHelp, Inc., and Heiler Software GmbH). With SigmaStat, statistical significance of differences between weight and height data of test plant groups was validated by using *t* test and Kruskal–Wallis one-way analysis of variance on ranks. Groups differing from the others were determined by use of an all pairwise multiple comparison procedure (Dunn's method).

ELISA for CMV quantification

Relative amounts of CMV (Le or Fny) in leaf samples were determined by direct double-antibody sandwich (DAS)-ELISA according to Dijkstra and de Jager (1998) using antiCMV antibody combination AS-0475 CMV (DSMZ, Braunschweig, Germany). Leaf material (0.2 g) of infected or control plants was homogenized in ELISA sample buffer (PBST ([140 mM NaCl, 2.7 mM KCl, 8 mM NaH_2PO_4, 1.8 mM KH_2PO_4 pH 7.4], 0.05% (v/v) Tween-20)+2% (w/v) polyvinylpyrrolidone-40 [Sigma PVP-40.000]) and kept at 4 °C overnight or up to 3 days. Following 5 min centrifugation (Eppendorf centrifuge, max. speed), serial dilutions of the samples were subjected to ELISA, using alkaline phosphatase-conjugated secondary antibody and *p*-nitrophenylphosphate substrate. A_{405} (versus A_{620}) was determined with BioRad ELISA plate reader model 3550. Dilutions of CMV particle preparations served as positive controls.

Viral nucleic acid detection and quantification by blot hybridization techniques

Tissue print blots, Southern blots (for AbMV detection) and Northern blots (for CMV RNA detection) were carried out to verify the infection status of single plants and individual leaves, and to compare relative amounts of viral nucleic acids between different groups of test plants. Tissue print blot methodology has been described (Morilla et al., 2004). Total nucleic acids were extracted from leaf samples (from 3 mm in diameter up to at maximum 10×20 mm) as follows: Tissue was frozen in 2 ml Eppendorf tubes in liquid nitrogen and ground by use of a glass rod. 0.5 ml homogenization buffer-(HB; 100 mM Tris–HCl pH 7.5, 1 mM Na-EDTA, 100 mM NaCl, 0.6% SDS, freshly supplemented with 100 mM DTT) and 0.5 ml phenol:chloroform (10:1 v/v, saturated with TE [10 mM Tris–HCl pH 7.5, 1 mM EDTA]) were added. Thawing and phenol extraction took place for 15 min at room temperature under vigorous shaking. Following 5 min centrifugation (Eppendorf centrifuge, full speed), the aqueous phase was removed and either re-extracted with phenol: chloroform as above or directly extracted with 1 vol. $CHCl_3$, mixed with 1/10 vol. 3 M Na-acetate pH 4.8 and 2 vol. ethanol (Rotisol, Carl Roth, Karlsruhe; − 20 °C), and kept on ice for 30 min to 2 h. Precipitated nucleic acids were sedimented by centrifugation, pellets washed with 70% ethanol (room temperature), dried and resuspended in 30 to 100 µl TE, or H_2O treated with dimethyldicarbonate (DMDC (Merck), 0.1% final concentration, degraded by autoclaving, according to the "DIG Application Manual for Filter Hybridization"; Roche Germany, Hoffmann-La Roche AG, Grenzach). Samples were run on agarose gels in TBE with 0.5 µg/ml ethidium bromide (EtBr) according to standard techniques (Sambrook and Russell, 2001), or, in order to separate glyoxylated RNA, in 1% agarose gels in 10 mM sodium phosphate buffer pH 7.0 supplemented with 10 mM sodium iodoacetate as described by Sambrook et al. (1989). Alkaline Southern blotting onto nylon membranes (Amersham

Hybond NX) followed the procedures of Chomczynski and Qasba (1984), neutral Northern transfer of samples to detect viral RNA was done according to Sambrook et al. (1989). All subsequent hybridization and detection procedures were the same for tissue print, Northern and Southern blots and used digoxigenin-labeled DNA probes (see below). Prehybridization and hybridization were carried out with minor modifications as described for radiolabeled probes by Sambrook and Russell (2001), using prehybridization solution (1% (w/v) glycine, 5× Denhardt's reagent, 5× SSPE, 0.3% (w/v) SDS, 0.1 mg/ml sheared fish sperm DNA, 50% (v/v) deionized formamide) for 3 h at 42 °C and 10 to 100 ng/ml heat-denatured digoxigenin-labeled probe (see below) in hybridization solution (10% (w/v) dextran sulfate, 5× Denhardt's reagent, 5× SSPE, 0.3% (w/v) SDS, 0.1 mg/ml salmon sperm DNA, 50% (v/v) deionized formamide) overnight at 42 °C. Posthybridization washes were 4× 15 min at 42 °C in 2xSSPE, 0.3% (w/v) SDS and 15 min in 0.2× SSPE at 65 °C. Chemiluminescent probe detection via CSPD or CDP-Star (Roche) followed the manufacturer's protocols (DIG Application Manual for Filter Hybridization; Roche).

For quantification of relative amounts of viral DNA in total plant nucleic acids, dilution series of two typical nucleic acid preparations from either an AbMV-infected *N. benthamiana*, or an infected tobacco plant, respectively, were analyzed on Southern blots also containing dilutions of hybridization standards (total cloned AbMV-DNA A fragments), and were detected the same way as all other blots, using both substrates for chemiluminescent probe detection (CSPD or CDP-Star) on separate blots. Variable exposures of X-ray films yielded equal band intensities of hybridization standards on "quantification blots" and "sample blots." Suitable blot pairs then allowed for direct estimation of relative AbMV DNA amounts between the different lanes on a "sample blot" in comparison to the hybridization signals on the "quantification blot", representing different volumes of a single AbMV-containing DNA isolation. To support optical estimations, X-ray films were scanned by use of a professional flatbed film scanner (CanoScan 9900 F), and average pixel intensities of representative single lane areas determined by help of SigmaScan Pro image analysis software (version 5.0.0, SPSS Inc.).

Viral nucleic acid in situ localization

AbMV DNA was localized in tissue sections of host plants by nonradioactive *in situ* hybridization (ISH), using a biotin-labeled probe detecting both AbMV DNA A and DNA B (see below). For *N. benthamiana*, leaf tissue samples from three independent co-infection experiments (two plants per type of infection, explants from young leaves up to 10 mm in length, and explants from fully expanded leaves 20 to 30 mm in length, containing class 2 or 3 veins centrally) were embedded in Paraplast plus paraffin embedding medium (Oxford, Sherwood Medical St. Louis, U.S.A.) at 42 dpai; for tomato, leaf samples from one representative co-infection experiment derived from two plants per type of infection (middle pinnate leaflet explants from young pinnate leaves [youngest and second youngest embeddable leaf], and from fully developed 3rd to 5th leaf separately, containing class 2 veins centrally) at 28 dpai and at 56 dpai. Leaf explants of CMV 3a protein-expressing *N. tabacum* plants, from leaves 3 or 7.5 cm in length (containing class 2 veins), or 15 cm in length (containing class 3 veins centrally) were chosen from a representative set of plants. Repeated preparations of comparable leaf samples from CMV 2b-transformed *N. tabacum* and the respective control lines were carried out at 42, 71, and 99 dpai, the latter 14 days after transfer of the plants to conditions of elevated light/temperature. If inoculated with CMV, only plants testing positive for CMV by ELISA and exhibiting a typical phenotype were processed. Formaldehyde fixation, embedding and sectioning procedures have been described in detail by Zhang et al. (2001). ISH, probe detection via nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) and microscopy were done as described by Morilla et al. (2004). In eight independent ISH experiments with 20 slides each (containing 20–50 sections from 1 or 3 explants per slide), specimens from 2 to 4 plants per type of infection and date of embedding were analyzed.

Hybridization probes

The full-length AbMV-DNA A insert of pDE3, detecting both AbMV genome components (Evans and Jeske, 1993), was isolated by digestion with *Pst*I and *Bgl*I, and gel-purified according to standard procedures (Sambrook and Russell, 2001). For filter hybridization, DNA was labeled with digoxigenin (DIG) by use of the DIG-High Prime labeling kit (No. 1 585 606, Roche, Germany) following the manufacturer's instructions. For ISH, probe biotinylation by nick-translation was carried out as described (Morilla et al., 2004). CMV RNA1, 2 and 3 were detected in Northern blots by cross-hybridizing DNA probes derived from K-CMV (Hellwald and Palukaitis, 1994) plasmids pK101 (containing full-length, but noninfectious mutant cDNA insert of RNA 1 in pUC18, kindly provided by Dr. K.-H. Hellwald), pK232 (infectious cDNA insert of RNA2 in pUC18; Hellwald and Palukaitis, 1994) and pK302 (infectious cDNA insert of RNA3 in pUC18; Roossinck et al., 1999). Full-length cDNA inserts were labeled with digoxigenin as above.

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