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Abutilon mosaic virus DNA B component supports mechanical virus transmission, but does not counteract begomoviral phloem limitation in transgenic plants

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Abstract

Different *Nicotiana benthamiana* lines stably transformed with *Abutilon mosaic virus* (AbMV) dimeric DNA B were capable of systemically spreading complete bipartite AbMV genomes, following agroinoculation of DNA A alone. Constitutively expressed viral movement protein (BC1) did not induce any persistent disease phenotype, but plants developed transient morphological abnormalities such as radially symmetric leaves after kanamycin withdrawal. Systemic AbMV infection produced symptoms and virus titers indistinguishable from those in non-transgenic plants. In systemically invaded leaves, the begomovirus remained phloem-limited, whereas the plants' susceptibility to mechanical transmission of AbMV was enhanced by a factor of three to five, as compared to non-transgenic controls. Hence, DNA B-encoded movement functions can complement local movement to the phloem after mechanical transmission, but fail to support viral invasion of non-phloem cells in systemically infected organs, indicating that the phloem restriction of AbMV does not result predominantly from a lack of transport competence in mesophyll tissues.

Keywords: Geminivirus; Begomovirus; *Abutilon mosaic virus*; Mechanical transmission; Movement; Transport; *In situ* hybridization; Tissue tropism; Phloem limitation; *Nicotiana benthamiana*

Introduction

Plant-pathogenic begomoviruses have attracted increasing attention during the last decades since they were shown to cause economically important newly emerging virus diseases, which are most pronounced in crop plants in tropical and subtropical areas as well as in the Mediterranean basin (Fargette et al., 2006; Mansoor et al., 2006, and references therein; Moriones and Navas-Castillo, 2000; Ribeiro et al., 2003; Rybicki and Pietersen, 1999). Although a few pathogens in the genus *Begomovirus* (family: *Geminiviridae*) were shown to harbor only a single circular single-stranded (ss)DNA genome component, encapsidated in particles of twinned ("geminate") shape, the majority of viruses in the genus have either bipartite genomes consisting of similarly sized DNAs A and B packaged separately, or are DNA A molecules accompanied by different types of satellite-like or subviral ssDNA agents (for review, refer to Briddon and Stanley, 2006; Stanley et al., 2005). Those DNA A-assisting molecules may mediate virus adaptation to new host plant varieties or species, and thus confer the begomoviruses remarkable capacities to spread as flexible disease complexes (Mansoor et al., 2003). At present, cotton, cassava, legumes, tomato and other crops in the *Solanaceae* are among the cultivated plants most heavily affected. Infectious virus particles are transmitted to host plants by the insect vector *Bemisia tabaci* Gennadius, a process which seems to involve structural interactions between viral coat protein domains and yet unidentified moieties of the whitefly (Böttcher et al., 2004; Ghanim et al., 2001; Höfer et al., 1997; Höhnle et al., 2001). These insects were shown to establish feeding sites on newly infested leaves by first moving their stylets through the apoplastic cell wall components of epidermal and non-vascular tissue layers, before they directly target and puncture phloem

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cells (Harris et al., 1996; Janssen et al., 1989; Pollard, 1955), which are sieve elements (SE), companion cells (CC) or cells in the vascular parenchyma (VP). Hence, in the natural situation begomoviruses will be delivered specifically into tissues which support replication of all geminiviruses analysed so far, and which later in the infection cycle mediate long-distance movement of the viral transport form (for reviews and details on the different stages of a systemic infection, refer e.g. to Nelson and van Bel, 1998; Waigmann et al., 2004; Wege, in press). After uncoating and initial replication, only certain begomoviruses egress from the vasculature into surrounding mesophyll tissues in primary infected or systemically invaded organs, whereas other members in the genus remain phloem-limited throughout the whole course of infection (Horns and Jeske, 1991; Morilla et al., 2004; Rojas et al., 2005; Wege et al., 2001, and references therein). Frequently, symptom severity correlates with the extent of tissue infiltration by the infectious agent (Wege et al., 2001). Tissue tropism may be host-dependent, and the molecular mechanisms underlying spread restrictions are diverse, even within a group of closely related viruses (as outlined in Wege and Siegmund, 2007). In addition, viral tissue specificity may be influenced by a number of distinct host and viral determinants at the same time, as exemplified by extensive complementation and recombination experiments exchanging genetic elements between the begomoviruses *Bean golden mosaic virus* (BGMV) and *Tomato golden mosaic virus* (TGMV; Morra and Petty, 2000). Several lines of evidence indicate that virus-encoded movement proteins (MPs), which function in cell-to-cell trafficking of viral transport forms, and ancillary virus-encoded proteins, are among the key players determining the tissue infiltration competence of a virus (Lucas, 2006). Hence, confinement to particular infection domains may in many cases at least partially result from dysfunction of viral MPs in other than the invaded cell types, or, alternatively, from a lack of operability only at the boundary between accessible and non-accessible cells (Ding et al., 2003; Lough and Lucas, 2006; Waigmann et al., 2004). Geminiviral MPs were shown to be major symptom determinants in systemic infections (Saunders et al., 2001, and references therein), although different results have been obtained with transgenic plants expressing BC1 proteins constitutively. Some of the respective plant lines exhibited disease-like phenotypes (Duan et al., 1997b; Hou et al., 2000; Pascal et al., 1993), whereas others grew normally and were indistinguishable from non-transformed plants (Hayes et al., 1988; Hussain et al., 2005).

For *Abutilon mosaic virus* (AbMV), a begomovirus which is strictly phloem-limited in all hosts analysed so far (DeSouza and Kim, 1990; Horns and Jeske, 1991, 2000; Jeske and Schuchalter-Eicke, 1984; Wege et al., 2000, 2001), we recently were able to show that co-infection with *Cucumber mosaic virus* (CMV; family: *Bromoviridae*) strongly enhanced the number of infected nuclei, and led to release of AbMV from the phloem into mesophyll parenchyma at several sites of highly infected leaves in different hosts. Transgenic plants revealed that the cucumoviral 2b silencing suppressor protein played an important role in the supportive effect, probably by complementing AbMV deficiencies in counteracting the host plant's silencing machinery (Wege and Siegmund, 2007). However, we could not rule out that further operative limitations of other AbMV proteins, namely its DNA B-encoded transport system consisting of the cell-to-cell MP (BC1) and the cooperating nuclear shuttle protein (NSP [BV1]; Frischmuth et al., 2007; Hehnle et al., 2004; Zhang et al., 2001), which mediates the intracellular transfer of viral nucleic acids from the nucleus to the cell periphery, contributed to phloem confinement of AbMV.

Therefore, we wanted to determine if the AbMV DNA B-encoded proteins are functional in mesophyll tissues in the absence of co-infecting, and possibly transport-complementing, supportive viruses. Thus, we transformed *Nicotiana benthamiana* leaf explant callus with dimeric AbMV DNA B, integrated in the T-DNA of a binary *Agrobacterium* plasmid vector, regenerated *N. benthamiana* lines and examined these plants for both complementation of infection with AbMV DNA A and differences in susceptibility to infection by sap inoculation of AbMV.

Results

Overview and experimental system

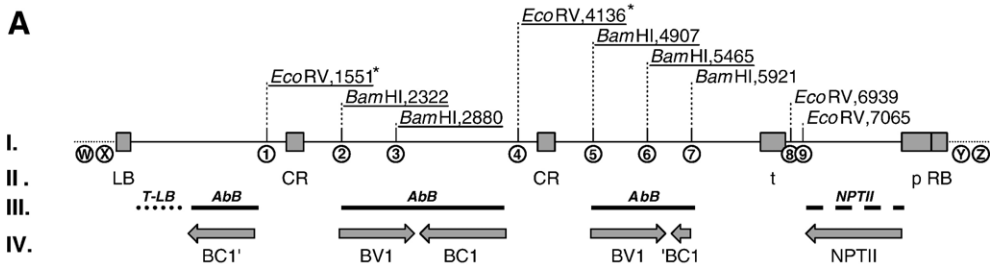
Progeny plants from independent transformants were monitored for AbMV DNA B-encoded protein expression and transgene-mediated phenotypic alterations. Generation T_4 progeny, which released circular DNA B copies upon replication of AbMV DNA A, was used to localize AbMV coat protein or nucleic acids in systemically invaded tissues by means of tissue blots or *in situ* hybridization, respectively. In order to detect functionality of the movement-associated AbMV proteins in non-vascular tissues of its host, we took advantage of the fact that phloem-limited viruses are barely mechanically transmissible by means of rub inoculation onto leaf lamina. This holds true also for plant species which usually are highly susceptible for mechanical virus transmission, including *N. benthamiana*. Similar findings have been reported for different phloem-limited geminiviruses such as *Tomato yellow leaf curl virus* (TYLCV; Avgelis and Roditakis, 2001; Makkouk et al., 1979), *Beet curly top virus* (BCTV; Bennett, 1934; Latham et al., 1997; Stanley et al., 1986), and BGMV (Kim et al., 1978) as well as e.g. for the phloem-confined *Citrus tristeza virus* (CTV; genus: *Closterovirus*, family: *Closteroviridae*; Garnsey et al., 1977). In contrast, mesophyll-invading geminiviruses like *Bean dwarf mosaic virus* (BDMV), *African cassava mosaic virus* (ACMV), and TGMV were shown to be readily mechanically transmissible to the same host species (Stanley, 1983; Stanley et al., 1990; Wang et al., 1996).

Furthermore, in some of the above studies on phloem-limited viruses, virus transmission efficiencies could be improved significantly by harsh procedures introducing inoculum directly into vascular cells. Therefore, gentle rub inoculation of leaf lamina is interpreted to target mainly epidermal and outer layers of mesophyll parenchyma cells (Nelson and van Bel, 1998, and references therein). By use of this method, we compared the susceptibilities of AbMV DNA B-transgenic plants for

mechanically applied virus inoculum to those of non-transgenic control plants.

N. benthamiana plants transgenic for dimeric AbMV DNA B exhibited transient kanamycin-dependent morphologic alterations

A dimeric head-to-tail clone of AbMV DNA B, inserted in the T-DNA of an *Agrobacterium* plasmid vector (Fig. 1), was introduced into *N. benthamiana* leaf callus by *Agrobacterium*-mediated transformation (Horsch et al., 1985). Several independent kanamycin-(kan)-resistant fertile plants were regenerated (generation T_1), tested by polymerase chain reaction (PCR) for the presence of AbMV open reading frames (ORFs) BC1 and BV1 (data not shown), and double-positive individuals were inbred to the 3rd or 4th generation (T_3/T_4) under repeated kan selection and PCR verification. A single non-resistant and PCR-negative line (line 14) was included in the first round of self-pollination. The organization of the AbMV DNA-containing T-DNA insertion was analyzed using T_4 progeny of randomly chosen lines 1, 7, and 10, by means of Southern blot hybridization analysis of total genomic DNA, following digestion with different restriction endonucleases as shown for plant line 7 in Fig. 1. Four enzymes yielding either characteristic



B

fragment	size (bp)
***Bam* HI**	
W → 2	2322 + w
2 → 3 5 → 6	**558** (2x)
3 → 5	**2027**
6 → 7	456
[7 → Y]	[2628 + y]
***Eco* RV**	
X → 1	1551 + x
1 → 4	**2585***
4 → 8	2803
[8 → 9]	[126]
[9 → Z]	[1484 + z]
***Nde* I**	
no internal	> 8549
***Xho* I**	
no internal	> 8549

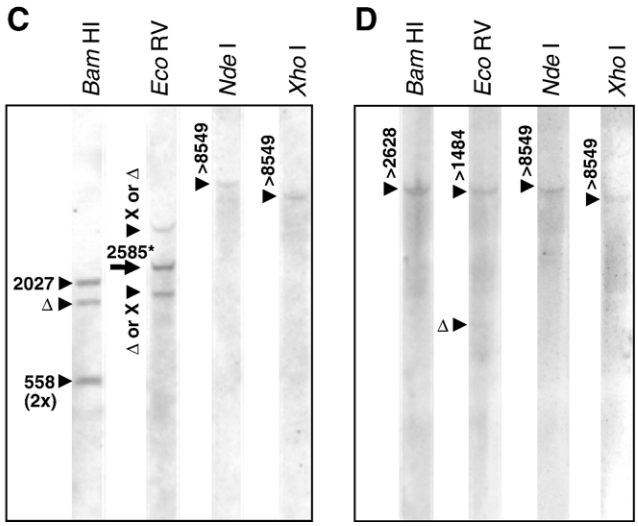


Fig. 1. Stable *Agrobacterium*-mediated transformation of a dimeric AbMV-DNA B construct into *N. benthamiana*. **(A)** Organization of a complete pBIN19 T-DNA carrying dimeric AbMV-DNA B (construct in total 8549 bp, integrated into plant DNA), **(I)** representation of DNA sequence including regulatory elements (grey boxes, specified in II), indicating numbered positions of characteristic restriction sites (W, X, Y, Z: hypothetic sites in adjacent plant genomic DNA). **(II)** Regulatory elements: *LB*: left border, *RB*: right border of T-DNA; *CR*: common region of begomoviral genome component, *t*: terminator/*p*: promoter [both originating from *nos* gene] regulating expression of *neomycin phosphotransferase II* (*npt II*) gene conferring kan resistance. **(III)** Target sequences hybridizing with three different construct-specific probes used in the analyses of different plant lines (*T-LB*: T-DNA sequence adjacent to left border, *AbB*: AbMV-DNA B region between BC1- and BV1-start codons, excluding large intergenic region/CR, *NPTII*: coding sequence of *npt II* gene). **(IV)** Positions of open reading frames. **(B–D)** Analysis of insert organization in transgenic *N. benthamiana* plants; **(B)** DNA fragments between numbered restriction sites, as expected for a single complete insertion of the T-DNA construct shown in panel A, following digestion with either of the restriction endonucleases indicated. Black: Fragments hybridizing with probe "AbB", grey: fragments not detectable with this probe. Bold and underlined fragment sizes were unequivocally detected in panel C, proving the identity of the transformed sequence at least between restriction sites 1 and 6: **(C)** shows Southern analysis of genomic DNA preparation of *N. benthamiana* line 7-12-I-T_4, using the indicated restriction endonucleases and probe "AbB". In panels B and C, asterisks label *Eco*RV fragment "1→4", representing the internal monomeric sequence of AbMV DNA B. Fragment sizes which are not underlined in B could not be identified in C, hence indicating deletions or rearrangements. Dotted lines correspond to fragments which may be present, but either could not be resolved in the analysis, or the size of which is not predictable, thus labeled with X in panel C. Δ: Bands indicative for deletions or rearrangements in C and D (see below). *Nde*I- and *Xho*I-generated fragments are underlined, since the sizes of the respective products point at a single insertion as detected in panels C and D. **(D)** Parallel blot, hybridized with probe NPTII, visualizing the rightmost fragments obtained with either *Bam*HI or *Eco*RV, respectively, or the complete inserted T-DNA construct in the case of the non-internally cutting enzymes *Nde*I and *Xho*I. DNA molecular weight markers present on the corresponding agarose gels verified the band size expectations indicated in C and D. All bands "> *n*" corresponded to fragments between 9240 bp (*Xho*I) and 12,460 bp (*Nde*I), as determined by use of the marker bands (not shown).

AbMV DNA-B-derived fragments (*Bam*HI and *Eco*RV), or lacking a recognition site in the T-DNA construct (*Nde*I and *Xho*I) were applied and the resulting products were blot-hybridized against three different probes as specified in Fig. 1A. The size of the fragments detected by a probe covering the open reading frames (ORFs) and the small intergenic region (SIR) of AbMV DNA B (Figs. 1A and B) verified that at least one complete DNA B insert flanked by two common regions (CR) was present in all three lines tested (Fig. 1B, underlined fragments, and C, and data not shown). In addition, both internally cutting enzymes produced single smaller fragments hybridizing to AbMV DNA B with any of the lines tested, indicating rearrangements or deletions close to the border region(s) of the insert (Δ in Fig. 1C). In most cases, the unique bands produced with *Nde*I or *Xho*I in combination with the AbMV probe (Fig. 1C), or with any of the enzymes in combination with a neomycin phosphotransferase (NPT)-specific probe hybridizing close to the right border (RB) region of the T-DNA (Fig. 1D), indicate a single copy insertion of the AbMV DNA B-derived construct in all lines analyzed, which seem to be accompanied by additional partial insertions. Genomic DNA fragments of the negative control line 14 did not hybridize with any of the probes used (data not shown).

All transgenic seedlings germinated on kan-containing selection plates completely arrested growth for 2 to 4 weeks after transfer to kan-free soil (data not shown). Subsequently, many of them developed one to a few abnormally elongated, distorted leaves up to an equifacial or even radially symmetric "*spikey*" phenotype (Fig. 2), separated by shortened internodes, in a slow and variable manner. About 2 to 4 weeks later, growth kinetics and leaf phenotype of all plants quickly recovered resulting in plants indistinguishable from wild type (wt) *N. benthamiana* (see also Fig. 3E). Transgenic seedlings raised on antibiotic-free plates grew continuously without any phenotypic alterations, like non-transgenic plants (wt and control line 14; data not shown). To exclude non-specific effects of kan release, two unrelated *N. benthamiana* lines expressing the same kan resistance gene, *npt II*, and either an AbMV DNA A-derived construct or a fragment of the bacterial *uidA* (β-glucuronidase) gene (Jefferson et al., 1986), both generated in our laboratory (unpublished), were germinated in parallel on kan-containing plates. They developed continuously and normally after transfer to soil (not shown), indicating that the transient alterations in plant development were specific for the AbMV DNA B transgene, but promoted by release of kan selection only. Light microscopy revealed aberrant tissue differentiation and a lack of polarity between upper and lower face for the leaf-like, spikey organs. They mainly consisted of isodiametric parenchyma cells, containing only rudimentary conductive tissues limited to the basal regions (Fig. 2$B_{1b/2b}$).

BC1 MP was expressed in all transgenic lines, but did not induce any disease-like phenotype in mature plants

To find out if the AbMV DNA-B-encoded ORFs BC1 and BV1 were constitutively expressed via their cognate promoters in the transgenic plants, and thus viral proteins might have been the cause of the observed abnormal phenotype, immunoblot analyses were carried out using protein preparations extracted at different stages of plant development. Since former results had shown that upon differential centrifugation of homogenates from infected plants, AbMV BC1 (MP) protein could be best detected in supernatants obtained by centrifugation at 20,000×*g* (abbreviated "S20"; Zhang et al., 2001), and about 50% of AbMV BV1 (NSP) protein partitioned in the same manner (unpublished data), S20 supernatants were tested for the presence of AbMV MP, or NSP, respectively, with rabbit antisera raised against heterologously expressed proteins (Fig. 3). Due to the low number of AbMV-infected cells, and the transient function of movement-associated proteins (Zhang et al., 2001), blots had to

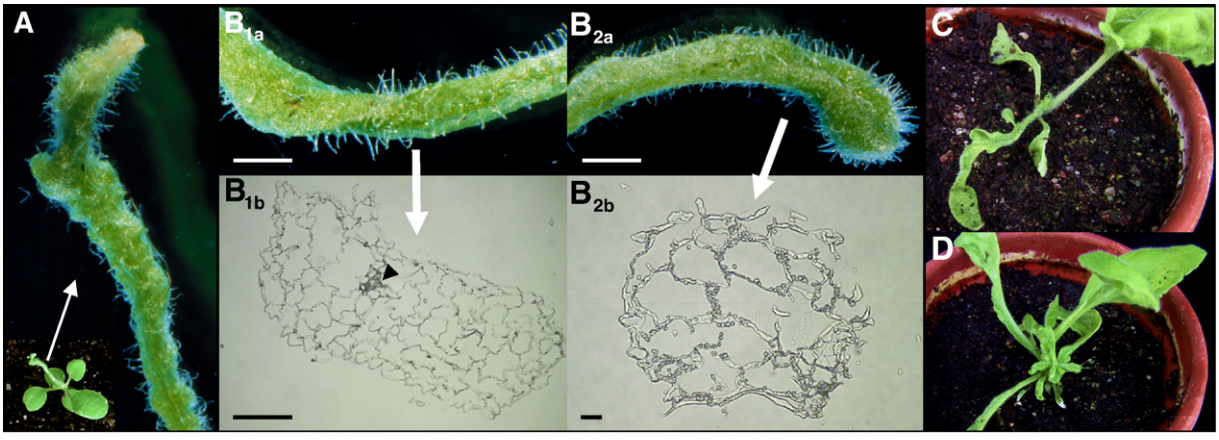


Fig. 2. Alterations of leaf morphology in young AbMV DNA B-transgenic *N. benthamiana* plants raised under kan selection. **(A)** Most extreme phenotype of a third true leaf, exhibiting "*spikey*"-shape, about 1 cm in length (of seedling from line 7 T_4 progeny, 21 days post planting, as shown in total in inset); **(B)** similar leaf (B_{1a}: basal; B_{2a}: tip region in detail; bars: 1 mm) and light microscopy of corresponding 7 μm cross sections (B_{1b}/B_{2b}: leaf regions indicated by arrows; bars: 50 μm/10 μm, after paraffin embedding and clearing). Arrowhead in panel B_{1b} points at central vascular elements, which are surrounded by parenchymatic cells lacking extensive differentiation. In the tip region (B_{2b}), no conductive tissues can be identified at all. (**C**, **D**) About 2 to 4 weeks later, upon growth recovery, plants develop increasingly normal leaf phenotypes, from a distorted, completely misshaped lamina up to slightly lanceolate and, finally, typical *N. benthamiana* shape. (C) Line 10, (D) line 7 progeny.

be over-exposed to reveal the presence of MP (as exemplified in Fig. 3B) or NSP (Figs. 3C, D) in systemically invaded *N. benthamiana* tissues, serving as positive control (lane 2 in Figs. 3A–D), against a background of plant proteins (lanes 1 and 3 in Figs. 3A–D, the cross-reactivity of which resulted from antibodies present in the pre-immune sera; data not shown). In transgenic plants from all three lines tested, different amounts of MP could be detected, irrespective of the age of the sampled plant. Transgenically produced MP migrated in a cluster of three bands, as for MP expressed during the natural infection, which suggests post-translational modifications including phosphorylation (Kleinow et al., unpublished data; Wege and Jeske, 1998). However, in no case did the amount of MP in transgenic tissues exceed that found in systemically infected comparable tissues, irrespective of the age of the source plant or the organ. Furthermore, no correlation was found between the MP level in individual plants and their respective actual or former phenotype, though the amount of protein extractable from the small spikey-shaped leaves was not sufficient for immunoblot testing.

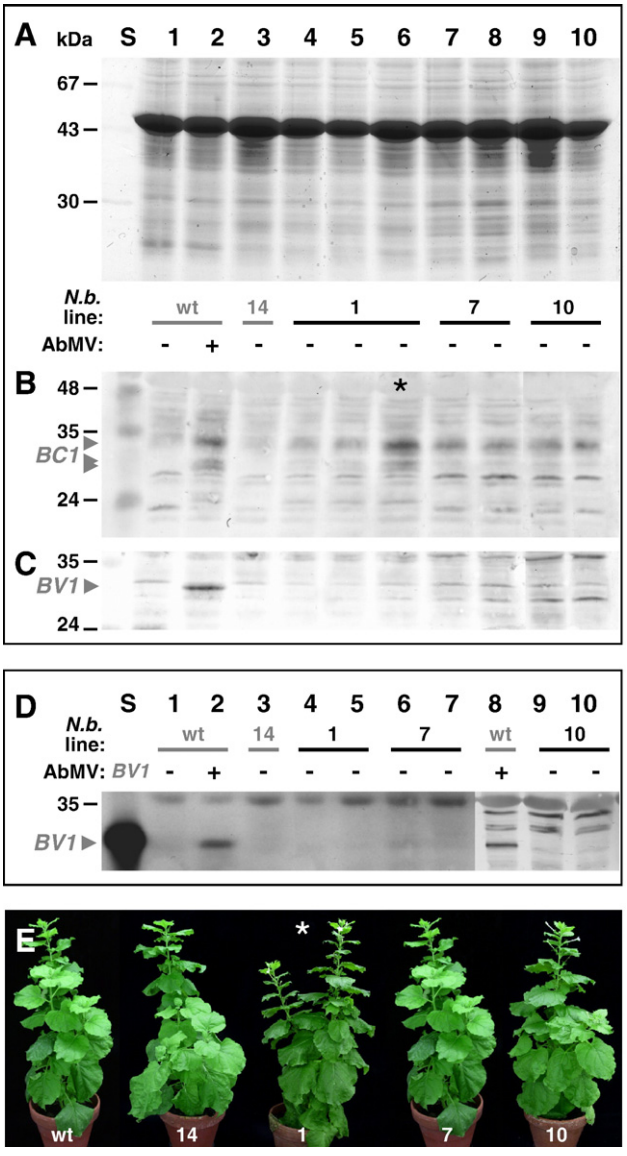


Fig. 3. Western blot analysis of AbMV DNA B-encoded protein expression in transgenic and control plants. **(A)** Total protein extracted from individual plants of *N. benthamiana* (enriched in 20,000×*g* supernatant fraction [S20]): uninfected or systemically AbMV-infected wild type control (wt; lanes 1 and 2, 23 dpi), control line 14 (generation T_2, lane 3), transgenic lines 1, 7, and 10 (all T_4, lanes 4–6, 7 and 8, or 9 and 10, respectively; lines 1 to 14: 8 weeks post planting). SDS–polyacrylamide (PA) gel (12.5%), Serva violet-17 stain. (S) Standard, M_r as indicated at left side. **(B, C)** Western blots of gels run in parallel, used for detection of B: AbMV–BC1 movement protein (MP), (C) AbMV–BV1 nuclear shuttle protein (NSP). Arrowheads (at the left) point at characteristic positions of BC1- or BV1-specific bands, respectively, which are most obvious in lanes 2 (AbMV-infected positive control). IgG detection via alkaline phosphatase (AP) and NBT/BCIP. Asterisk in B denotes the same plant as shown in E/1, expressing significant amounts of MP. **(D)** Western analysis for the presence of AbMV NSP in S20-enriched proteins of the *N. benthamiana* lines indicated, using different enhanced-sensitivity protocols after separation in 15 % SDS–PA gels. Lanes S to 7: Proteins from negative and positive controls as above, and from T_4 plants in lines 1 and 7, following IgG detection by use of "enhanced chemiluminescence" (ECL, horseradish peroxidase [HRP]-based). Lane 8 (AbMV-infected positive control) to 10 (9/10: 2 plants from line 10): total S20-enriched proteins extracted in the presence of high amounts of proteinase inhibitors (wt+AbMV: PMSF, lane 9: PMSF+Sigma protease inhibitor cocktail, lane 10: PMSF+Roche EDTA-free protease inhibitor cocktail), lanes 8–10 treated with iodoacetamide prior electrophoresis. (S) *E. coli*-expressed BV1 protein. **(E)** Phenotype of control or AbMV DNA B-transgenic *N. benthamiana* plants (the lines of which are indicated). Wt and line 14: 10 weeks, lines 1, 7, 10: 14 weeks post planting. Plants are phenotypically identical siblings of the ones probed for AbMV protein expression (as shown in panels xB to D), except the plant from line 1 (asterisk) which is the one used for protein extraction for the above analysis. None of the plants exhibits any obvious abnormalities.

Thus, we cannot exclude a transient peak of virus protein production in those organs. Fig. 3E shows mature plants, which after initial morphological disturbances were growing without any phenotypical alterations or disease-like symptoms, but constitutively expressed BC1 MP up to levels comparable to those in an AbMV systemic infection (asterisks). By contrast, no BV1 NSP could be detected by immunoblot analyses of protein from expanded leaves and apical tissues from any of the transgenic plants analysed, despite several efforts to improve extraction and detection procedures, including addition of different protease inhibitors and iodoacetamide-mediated alkylation of proteins (Görg et al., 1987; Lane, 1978) prior to electrophoresis (Figs. 3C, D, and data not shown). In addition, because NSP was shown to accumulate in nuclei of infected cells (Zhang et al., 2001), nuclei were isolated from noninfected transgenic, and from noninfected as well as AbMV systemically infected wt *N. benthamiana*. Neither immunoblot analysis with protein extracts prepared from the nuclei nor *in situ* immunology with intact nuclei fixed on slides revealed any evidence for constitutive or temporal expression of detectable amounts of NSP in the transgenic plants (data not shown).

Following DNA A inoculation, circular AbMV DNA B components were released from the genomically integrated copies, resulting in typical AbMV infections

AbMV DNA B dimer-transgenic plants were agroinoculated (Klinkenberg et al., 1989) with either single or both

AbMV DNA components (Frischmuth et al., 1993) and the resulting effects monitored by optical rating and Southern blot analyses of total nucleic acids isolated from newly developed leaves (Fig. 4). Whereas in none of the plants inoculated with AbMV DNA B alone did any symptom develop, as was expected for transgenic as well as non-transgenic control plants, *Agrobacterium*-mediated delivery of DNA A alone led to symptom formation in the inoculated plants in lines 1, 7, and 10. Symptom development was typical of AbMV-infected *N. benthamiana*, resulting in relatively mild leaf curvatures and lamina wrinkling, inconspicuous slightly brightened green patches and chlorosis, and limited stunting. Symptomatology was indistinguishable from that in non-transgenic control lines 14 and wt plants inoculated with DNA A and B, and occurred after similar latent periods of 8 to 12 days. Agroinfection of the transgenic plants by use of both AbMV genome components induced the same phenotype. Newly formed expanding leaves were collected from every plant in the experiments and the extracted total nucleic acids were examined for the presence of free AbMV DNA B and DNA A by Southern blot analysis with AbMV DNA B-, or DNA A-specific probes, respectively. As shown in Figs. 4A and B, double-stranded (ds) circular as well as ssDNA B of monomeric size was readily detectable in all symptomatic plants; i.e., control and transgenic plants inoculated with both viral genome components, and all DNA B-transgenic ones inoculated solely with AbMV A. Hence, freely replicating copies of the tandemly inserted AbMV DNA B had been released from *N. benthamiana* genomic DNA after introduction of AbMV DNA A. Approximately 50% of the DNA A-inoculated non-transgenic control plants were systemically invaded by AbMV DNA A molecules, predominantly consisting of ssDNA, in the absence of symptoms, confirming former findings for AbMV (Evans and Jeske, 1993; Haible et al., 2006) and ACMV (Klinkenberg and Stanley, 1990). During the course of the experiments, none out of 20 DNA B-agroinoculated transgenic plants accumulated detectable levels of free DNA B, and none of the generation T_4 plants had lost the stably integrated DNA B construct since it was released in 100% of the DNA A-inoculated individuals.

Due to the deviating growth kinetics of transgenic and control *N. benthamiana*, agroinfection of the controls had to be carried out with younger plants of developmental stages comparable to those of the transgenic ones. In some of the experiments, this led to slight variability in the onset and thus progress of viral replication between control and transgenic groups, as it is obvious for wt plants in Fig. 4, in contrast to the non-transgenic plants in line 14. For this reason, we did not try to exactly quantify viral DNA levels in the different plant types. However, comparison of all Southern blots prepared after independent agroinfection experiments suggested that no significant differences existed in AbMV DNA accumulation between transgenic and non-transgenic *N. benthamiana*, irrespective of whether

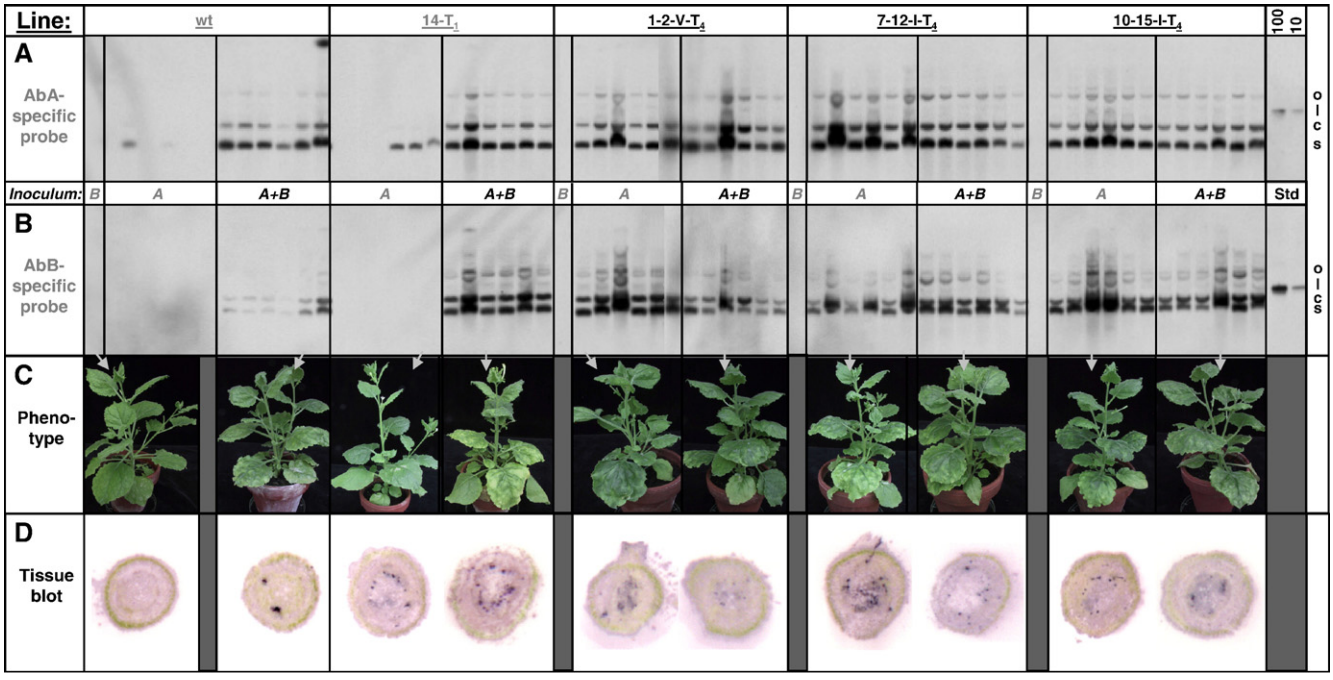


Fig. 4. Viral genome component accumulation in systemically invaded leaves, and symptomatology in control and AbMV DNA B-transgenic plants (*N. benthamiana* lines as indicated in the top row), following agroinoculation with single or both AbMV components as specified (row "inoculum"). **(A, B)** Southern blot analysis showing accumulation of AbMV DNA A (A) or B (B) in total nucleic acid samples extracted from young leaves of individual sibling plants at 30 dpi; main AbMV DNA forms are indicated (right column; o: open circular, l: linear, c: covalently closed circular, s: single-stranded; separation in 1% agarose in TBE in the presence of ethidium bromide). Std: Hybridization standards, 100 or 10 pg of linear AbMV DNA A or B. **(C)** Phenotype of representative plants in the analysis at 47 dpi; arrows indicate the plants analyzed in the above Southern blot lanes (no wt plant inoculated with AbMV A is shown). **(D)** Immunological NBT/BCIP-based detection of AbMV coat protein (CP) in tissue prints of stem cross sections from the plants shown above (or from a sibling in the respective group). Dark bluish spots represent CP accumulation, violet and greenish areas are caused by non-specific background staining and plant compounds.

inoculated with DNA A alone in the case of transgenic, or DNA A and B in transgenic or control plants, respectively (Fig. 4, and data not shown).

In systemically infected tissues, the phloem-associated distribution of AbMV coat protein and DNA did not differ between non-transgenic and transgenic plants

To determine whether the presence of dimeric AbMV DNA B alters the tissue specificity of systemically spreading AbMV, tissue blots of all plant line/inoculum combinations described above were tested for the distribution of AbMV coat protein (CP; exemplified by Fig. 4D). Freshly prepared cross sections of stems, petioles and furled leaves were gently printed onto nitrocellulose membrane, dried, and used for immunodetection of AbMV CP with rabbit antisera. In most of the prints from systemically invaded plant organs of any genotype, AbMV antigen was predominantly detected in association with veins or the vascular cylinder inside the stem (Fig. 4D), as shown by bluish NBT/BCIP precipitates. Occasionally, however, signals also covered or leaked into pith or cortical regions of the stem, or apparently non-vascular portions of leaf blots, making interpretation difficult. Therefore we decided to analyze the distribution of AbMV nucleic acids in specimens from systemically infected tissues of transgenic and control plant lines by means of *in situ* hybridization at the light-microscope level. Paraffin-embedded explants from highly symptomatic, fully expanded leaves of systemically infected wt plants, transgenic plants from lines 1 and 10, and DNA B-inoculated controls were sectioned and subjected to hybridization with a biotinylated probe detecting both AbMV DNA A and B via precipitation of a blue tetramethylbenzidine (TMB)-derived stain. A total of about 650 leaf cross sections derived from line 10 (T_4), and about 350 sections each from line 1 (T_4) and wt plants revealed that AbMV DNA was confined to phloem cells close to vascular elements in all plant types tested (Fig. 5). DAPI counterstaining showed that signals typically labelled nuclei. No obvious differences were observed between transgenic and non-transgenic leaf lamina with respect to the average numbers of infected nuclei per mm section length (data not shown).

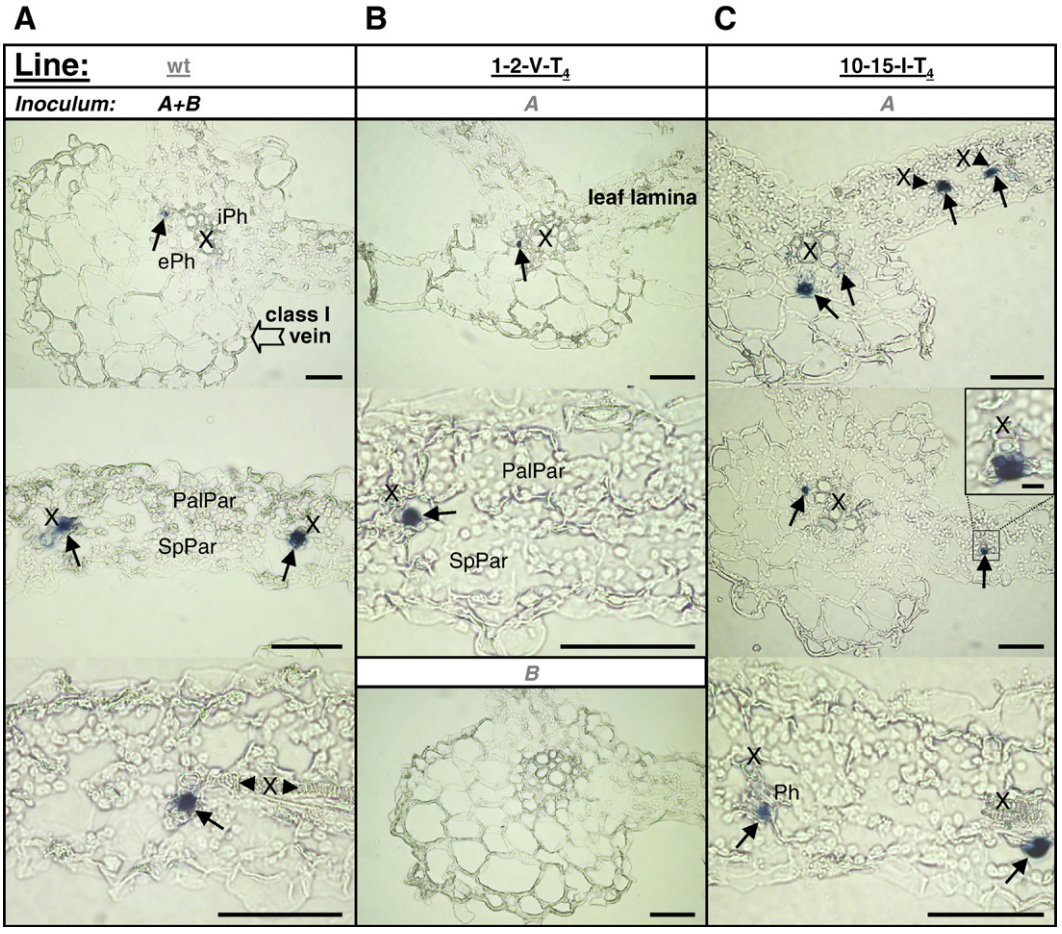


Fig. 5. *In situ* detection of AbMV DNA in cross sections of systemically infected, fully expanded symptomatic leaves from **(A)** wt *N. benthamiana* agroinfected with AbMV DNA A and B, **(B)** AbMV DNA B-transgenic plants in line 1 (generation T_4), inoculated with AbMV DNA A, or AbMV DNA B for control (bottom), **(C)** AbMV DNA B-transgenic plants in line 10 (T_4), inoculated with AbMV DNA A. Inset shows magnification of the outlined area. Prior embedding, all plants (except controls) were shown to multiply free DNA A and B by Southern analysis. *In situ* AbMV DNA detection via TMB resulted in blue signals (arrows). Brightfield microscopy; X (some in conjunction with arrowheads): xylem, (e/i)Ph: (external/internal) phloem, PalPar: palisade parenchyma, SpPar: spongy parenchyma (representative marking). Scale bars: 50 μm.

Plants transgenic for AbMV DNA B exhibited greatly enhanced susceptibility to mechanical transmission of AbMV

In the past, many attempts to mechanically transmit AbMV between *N. benthamiana* plants have failed (Jeske, 2000, and unpublished data). Inefficient mechanical transmissibility to otherwise readily susceptible hosts also has been described for a number of different phloem-limited viruses and seems to reflect the fact that rub inoculation onto leaf lamina usually targets outer tissue layers such as epidermis and mesophyll parenchyma, as specified in the Introduction. We therefore sought to determine if the ubiquitous presence of translatable DNA B copies, which may be mobilized by AbMV DNA A, might increase the plants' susceptibility for mechanical virus uptake by enabling active AbMV passage through non-vascular cells. Therefore, initially, mechanical transmissibility of AbMV from systemically infected wt *N. benthamiana* to uninfected plants of the same genotype was re-analyzed. Whereas no transfer of virus via water- or buffer-diluted source plant extracts was possible, transmission occasionally succeeded when systemically infected leaves were freshly cut, squeezed and the edges were drawn gently over Carborundum-dusted upper epidermis of the target plants' leaf lamina. Handling was successfully optimized to achieve only exceptional, but reproducible transmission events, which suggested that under the respective conditions, vascular tissues were usually not directly inoculated, but the sap inoculum was infectious. Subsequently, a series of experiments treating non-transgenic and AbMV DNA B-transgenic plants from all lines in parallel was carried out, the results of which are shown in Table 1. In comparison to wt *N. benthamiana* or control line 14, plants in all three transgenic lines exhibited a significantly enhanced susceptibility for sap-inoculation with AbMV. In contrast to the control plants of which about 6% developed productive virus infections, the percentage of successful transmission events was increased by a factor of about three to five: Depending on the individual transformant line, transmission was achieved to an average percentage between 16 and 31% of the inoculated plants. χ^2 testing verified that the observed differences were statistically significant. AbMV DNA A- and DNA B-specific Southern hybridization analysis was carried out with total nucleic acids isolated from newly developed leaves of all mechanically treated plants, and the formation of disease symptoms was monitored (data not shown). In comparison to agroinfected seedlings, the onset of symptoms was delayed by at least 10 days in all successfully inoculated plants, starting between 3 and 5 weeks, occasionally up to 8 weeks, post inoculation, resulting in the characteristic phenotype of AbMV infections as described before. In any fully symptomatic plant analyzed, similar titers of both viral DNA components were detectable which did not differ from those accumulating after agroinoculation. Non-transgenic plants in which mechanical AbMV inoculation had succeeded, developed symptoms more slowly than most of the transgenic plants treated in parallel, and accumulated reduced levels of viral DNA (data not shown).

Table 1
Mechanical transmission of AbMV via sap from systemically infected wt *N. benthamiana* to control (i.e. wt and 14-$T_{1\ or\ 2}$) or independent AbMV DNA B-transgenic lines

Experiment/Line	wt	14-$T_{1\ or\ 2}$	1-2-V-T_4	7-12-I-T_4	10-15-I-T_4
1	2/10 (20)	2/17 (12)	n.d.	n.d.	10/20 (50)
2	0/15 (0)	0/17 (0)	5/18 (28)	3/25 (12)	1/11 (9)
3	0/15 (0)	1/15 (7)	2/15 (13)	3/15 (20)	5/15 (33)
4	1/24 (4)	n.d.	n.d.	n.d.	n.d.
Mean %	6	6	21	16	31
SD	±9.5	±6.0	±10.6	±5.7	±20.6
Total *	3/64 (5)	3/49 (6)	7/33 (21)	6/40 (15)	16/46 (35)

Data indicate systemically infected/out of inoculated plants (percentage of infected plants); the respective infection status was determined by Southern hybridization analyses. In all infected plants, AbMV DNA A as well as DNA B were present.
SD: standard deviation; "n.d.": not determined.
* Statistically significant differences between either group of control plants and any of the transgenic plant lines, for $\alpha < 0.05$ as validated by χ^2 test.

Discussion

N. benthamiana was stably transformed with tandemly repeated AbMV DNA B components. To our knowledge, this is the first report on plants transgenic for the DNA B of a phloem-limited geminivirus. Despite expression of MP from the constructs, mature plants did not exhibit disease-like phenotypes. However, following release of kanamycin (kan) selection, seedlings underwent severe transient growth disturbances resulting in stunting and elongated up to spikey, radially symmetric leaf morphology, lacking polarity between adaxial (upper) and abaxial (lower) face. Interestingly, the phenotype strongly resembled that of transgenic *N. benthamiana* expressing the first protein in the triple gene block (TGB1) of *White clover mosaic virus* (WClMV), a potexvirus (Foster et al., 2002). TGB1, in combination with TGB2 and 3, is crucial for the cell-to-cell movement of WClMV RNA–CP complexes, binds RNA, increases the size exclusion limit (SEL) of plasmodesmata, traffics from cell to cell, and suppresses RNA silencing (Bayne et al., 2005; Lough et al., 1998; Lucas, 2006; Verchot-Lubicz, 2005; and references therein). Foster et al. (2002) considered that in the apices of distorted plants, TGB1 transport functions competed with plant non-cell-autonomous proteins (NCAP) or NCAP/ribonucleoprotein complexes (RNPC), impairing surveillance and/or signal distribution in the RNA-based information system which is involved in the establishment of developmental patterns and organ polarity (reviewed by Ding et al., 2003; Lough and Lucas, 2006). It may also interfere with Argonaute (ago) proteins which contribute to polarized leaf morphogenesis by tissue-specific mRNA cleavage (Baumberger and Baulcombe, 2005; Bayne et al., 2005; Foster et al., 2002; Jones et al., 2006; Kidner and Martienssen, 2004, 2005; Voinnet et al., 2000).

Although no influence of AbMV BC1 MP on the plant's silencing machinery has been described, it shares a number of functional analogies with TGB1 though it harbours a membrane-anchoring helix, which is absent from the potexviral MP (Aberle et al., 2002; Frischmuth et al., 2004; Zhang et al., 2002). AbMV MP exhibits a limited nucleic acid binding capacity, forming

characteristic NPCs in the presence of additional AbMV NSP (Hehnle et al., 2004). It targets the cell periphery where it accumulates at sites which may be associated with plasmodesmata (Zhang et al., 2001). In the presence of cognate viral DNA and NSP, which the MP redirects from nuclei to the cell cortex (Frischmuth et al., 2007, and references therein; Sanderfoot and Lazarowitz, 1995), both MP and NSP co-traffic through plasmodesmata into adjacent cells in sink leaves of host plants (Zhang et al., 2001). Thus, movement of viral nucleic acid is obviously mediated by a concerted action of both proteins, as it has been deduced from several lines of evidence also for the phloem-limited begomovirus *Squash leaf curl virus* (SLCV, formerly SqLCV; reviewed by Lazarowitz, 1999).

In analogy to the interpretation for WClMV TGB1, distortions in plant morphology and leaf polarity might arise from an accumulation of constitutively expressed AbMV MP in membranes close to plasmodesmal docking sites and SEL complexes (Lucas, 2006), hindering cellular communication. The fact that the migration behaviour of transgenically expressed AbMV MP in SDS–PAGE indicated correct modification might support this suggestion. MP levels were similar to those in natural infections, which might correspond to an accumulation of moderate amounts in a high number of cells in the transgenic *N. benthamiana*, as opposed to the production of large quantities in a low number of cells during systemic AbMV movement, but was not analyzed in more detail. Since we could not extract sufficient amounts of protein from spikey leaves to allow for MP quantification, we were not able to investigate if a positive correlation between transgene expression and the extent of morphological disorders existed. After growth recovery, no such correlation was observed. Interestingly, the AbMV DNA B-dependent distortions were triggered only after release of kan selection. We propose that transient metabolic disorders during kan withdrawal perturbed an otherwise balanced dynamic metabolic state which probably involved continuous MP detoxification. This idea is supported by the fact that the aminoglycoside antibiotic kan preferentially interferes with ribosomal function in mitochondria and plastids, but also impedes cytoplasmic translation to some extent (Kotra et al., 2000). Consequently, after its release, overall protein expression including that of AbMV MP should be enhanced. Plants might need a few weeks to adapt and counteract or balance toxic effects henceforth: All mature transgenic plants were asymptomatic. This corresponds with previous findings on tobacco and petunia transgenic for TGMV DNA B di- or multimers, both of which looked normal, though obviously in any developmental stage (Hayes et al., 1988; Rogers et al., 1986). Tobacco stably transformed with a construct designed for *Tomato leaf curl New Delhi virus* (ToLCNDV) BC1 expression under control of the CaMV 35S promoter (Hussain et al., 2005) also was symptomless. In all of those studies, however, ectopic protein levels were not analyzed. By contrast, transgenically expressed full-length BC1 MPs of some other begomoviruses were shown to induce morphological alterations as well as disease-like symptoms: *Tomato mottle virus* (ToMoV) MP produced in tobacco (Duan et al., 1997a, 1997b), *Bean dwarf mosaic virus* (BDMV) MP in tomato (Hou et al., 2000), and SLCV MP in *N. benthamiana* (referred to as tobacco; Pascal et al., 1993). Since in those studies significant amounts of the respective MPs were detected in the transgenic tissues, pathogenic phenotypes may depend on the level of ectopic protein expression. Alternatively or additionally, the differences may reflect that begomoviral BC1 MPs fall into distinct classes, distinguished by their molecular modes of action, which is supported by a number of discrepancies between experimental data on nucleic acid binding and cell-to-cell trafficking capacities of MPs and NSPs from individual begomoviruses (Frischmuth et al., 2007; Hehnle et al., 2004; Rojas et al., 2005, and references therein; Ward et al., 1997). Unlike AbMV MP, no NSP was detectable in any DNA B-transgenic plant in all lines analyzed, which is in line with previous analyses on different begomoviruses showing dependence of BV1 expression on transactivation by DNA A-encoded AC2 protein (e.g. Shivaprasad et al., 2005; Sunter and Bisaro, 1992). Two studies on *African cassava mosaic virus* (ACMV) observing high basal activities of its BV1 promoter (Frey et al., 2001; Zhan et al., 1993) may point at virus-specific differences.

Agroinoculation of AbMV DNA B-transformed *N. benthamiana* with both AbMV genome components resulted in typical systemic infections with respect to viral DNA titers and symptoms. Inoculation of AbMV DNA A alone led to release of freely replicating DNA B and ended in full infections indistinguishable from those after initial delivery of DNAs A and B. These results are consistent with two earlier sets of studies, both of which used plants transgenic for tandem copies of TGMV DNA B, either systemically infectable (*Nicotiana tabacum* and *N. benthamiana*) or not (petunia) with TGMV (Hayes et al., 1988, 1989; Rogers et al., 1986; Sunter et al., 1987). As in our experiments, DNA B molecules were released from the chromosomal copies after DNA A was delivered either by agroinfection (Hayes et al., 1988), by mechanical inoculation of DNA A-containing virions (Sunter et al., 1987), or by a cross with a tandem DNA A-transgenic plant (Rogers et al., 1986). As opposed to AbMV, TGMV invades all tissues of host plants' leaves (Saunders et al., 2001), for which reason the previous studies did not focus on virus distribution inside plant organs. Production of *N. benthamiana* transgenic for tandem repeats of AbMV DNA B has now allowed for testing the contribution of a begomoviral DNA B component to viral tissue tropism and/or mechanical transmissibility for the first time.

Notably, also in these plants containing translatable movement-associated ORFs BC1 (MP) and BV1 (NSP), which may be amplified via replicational release of circular DNA B in every cell, AbMV remained confined to the phloem during systemic infection. By contrast, a three- to fivefold increased susceptibility of the plants for mechanical AbMV uptake via the leaf surface suggested that the transgene supported trafficking of viral nucleic acids through non-vascular cells into the phloem in the inoculated organ. Hence, our experiments indicate that the transport as well as the replication systems of the cognate pair of AbMV DNA circles are functional in mesophyll parenchyma of *N. benthamiana*, although the virus does not invade this tissue in systemic infections. In contrast, in a bean cultivar not systemically infectable by AbMV, its DNA B did not traffic the homologous DNA A between non-phloem cells, but operated in

transport of co-inoculated BDMV DNA A (Levy and Czosnek, 2003). Our experiments, however, do not rule out that in systemically invaded leaves, a specific unidirectional transport barrier exists for AbMV at the boundary between vascular and non-vascular cells, which may be due to plasmodesmata or plasmodesmata-interacting proteins associated with bundle sheath or adjacent cells (Ding et al., 2003; Lough and Lucas, 2006; Lucas, 2006; Waigmann et al., 2004; Wege, in press; and references therein; Zhang et al., 2001). Recently, we were able to show that unrelated MPs of tobamo- or cucumoviruses (Pohl and Wege, 2007; Wege and Siegmund, 2007) were dysfunctional in gating AbMV into mesophyll tissues. Taken together, our findings therefore suggest a major role of DNA A-encoded functions in viral tissue restriction. This may involve the replication–cell cycle connection mediated by (A)C1 (replication-associated Rep protein; Kong et al., 2000) and/or (A)C3 (replication enhancer protein REn; Settlage et al., 2001, 2005), as well as non-coding regions (Morra and Petty, 2000). Co-infection and complementation experiments with CMV, or CMV 2b protein, respectively, lead to our former suggestion that AbMV might exhibit a lack in suppression of mesophyll-specific RNA silencing (Wege and Siegmund, 2007). In that case, AC2 and AC4 proteins of begomoviruses were the main candidates for proteins involved in this context (reviewed by Bisaro, 2006). In a complementary study on the largely phloem-limited luteovirus *Potato leaf roll virus* (PLRV), a gain in mechanical transmissibility to wt *Nicotiana* spp. was only observed if the inoculum contained a recombinant virus complementing functions in both silencing suppression and movement (Ryabov et al., 2001). For *Turnip crinkle virus* (TCV; family *Tombusviridae*) it was demonstrated that Dicer-like (DCL) 4 protein involved in RNA silencing represented the only and essential barrier against vascular exit of the virus in *Arabidopsis*; its inhibition by the TCV suppressor P38 mediated viral egress from the phloem (Deleris et al., 2006). If indeed and by themselves tissue-specific RNA silencing suppression characteristics may account for the phloem limitation of AbMV in systemic infections, or if localized spread interruptions at the vascular sheath are additionally involved, therefore remains to be determined. The plants transgenic for dimeric AbMV DNA B now represent a valuable tool for further analyses on phloem-restricted initiation of virus-induced gene silencing (VIGS; Krenz and Jeske, unpublished data to be published elsewhere).

Materials and methods

Plant transformation, propagation, and phenotype/symptom documentation

Non-transformed and mature transgenic *N. benthamiana* Domin were cultivated in an insect-free S2 containment greenhouse (16 h light [100 kW h] at 25 °C/8 h dark at 20 °C; 60% relative humidity). Following 24 h of callus induction on Murashige and Skoog growth medium (MS; Murashige and Skoog, 1962; Sigma no. 5524 supplemented with vitamins and phytohormones) solidified with 1% (w/v) agarose, wt leaf explants were transformed with dimeric AbMV DNA-B inserted in the T-DNA of pBIN19 (Bevan, 1984; clone AbB described in Frischmuth et al., 1993) by use of *Agrobacterium tumefaciens* LBA 4404 (Hoekema et al., 1983), and transformed shoots regenerated and selected in the presence of 100 mg/l kan essentially as described (Horsch et al., 1985), giving rise to 12 independent fertile transgenic and a single non-transgenic plant (no. 14) referred to as "generation T_1". Seeds from plants tested positive for the presence of both ORFs BC1 and BV1 by PCR were sterilized, germinated on MS medium supplemented with 500 mg/ml kan, transferred into soil and kept in a humid cultivation box for a few weeks, until growth of phenotypically normal leaves indicated adaptation to kan-free conditions. Inbreeding to the 3rd or 4th generation (T_3/T_4) took place under repeated kan selection and PCR verification. The kan-non-resistant, PCR-negative line 14 was included in the first round. T_4 sibling plants analyzed in this study stem from self-pollinated parents 1-2-V, 7-12-I, and 10-15-I. Phenotype or symptoms were monitored at least every 2 to 3 days and photographs taken either on Kodak Ektachrome 50 film with an Olympus OM4-Ti, or with a Canon PowerShot G1, or G5, camera.

Virus inoculation

AbMV was introduced into plant seedlings in the two-to-four-leaf stage via stem agroinoculation (Klinkenberg et al., 1989) using clones AbA (AbMV DNA A) and AbB (AbMV DNA B; Frischmuth et al., 1993) in *A. tumefaciens* LBA 4404 (Hoekema et al., 1983). Mechanical transmission of AbMV from agroinfected source plants was done as follows: Fully expanded, highly symptomatic leaves from upper stem regions (2nd to 4th expanded leaf, positions at the plants and symptoms as similar as possible) were freshly cross-cut, rolled up, squeezed, and the cut edges were drawn gently over the upper epidermis of two independent Carborundum (600 mesh)-dusted young, but completely unfolded leaves of target plants harboring 3–5 expanded true leaves. About 1 min later, the plants were rinsed with tap water removing Carborundum and excess inoculum.

Immunoblot analyses

Protein extraction from young leaves up to 15 mm in length was carried out as described (Zhang et al., 2001), and the homogenate centrifuged for 5 min at 20,000×*g* (4 °C) to yield supernatant "S20". In some experiments, the extraction buffer was supplemented with a protease inhibitor cocktail (Sigma no. P9599, or Roche no. 1873580, respectively, to final concentrations recommended by the supplier). Discontinuous SDS polyacrylamide gel electrophoresis using protein molecular weight standards from Amersham/GE Healthcare (Electrophoresis Calibration Kit for low molecular weight proteins) or MBI Fermentas (Prestained Protein Ladder 10–180 kDa), protein transfer to nitrocellulose membrane, gel and blot staining, and immunodetection procedures were performed as specified in Zhang et al. (2001). Polyclonal rabbit antisera detecting AbMV MP or NSP were raised commercially (pab productions, Hebertshausen, Germany) against purified, bacterially expressed antigens (Wege and Jeske, 1998). Prior to the

first immunization dose, pre-immune sera were collected from every treated animal. Sera from different blood samples taken after consecutive boost immunizations were tested for reactivity and specificity on Western blots with total protein from a number of AbMV systemically infected host species and compared to samples from uninfected plants and from antigen-expressing bacteria. In the experiments in this study, suitable sera were diluted 1:1000 (anti-BV1) or 1:5000 (anti-BC1), respectively, and primary antibodies detected via alkaline phosphatase-conjugated affinity-purified goat anti-rabbit IgG (Rockland Inc., Gilbertsville, USA). In a number of experiments, horseradish peroxidase-conjugated secondary antibody was used in combination with the "Enhanced chemiluminescence (ECL) detection system" according to the manufacturer's instructions (GE Healthcare). Tissue print blots prepared by gently pressing freshly cut leaf, petiole, and stem cross sections onto nitrocellulose membrane (PROTRAN BA 85, Schleicher & Schuell) were analyzed with identical detection procedures, using nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) as substrates.

Nucleic acid preparation, restriction digestion, and detection by blot hybridization techniques

To determine the transgene status of selected plants, Southern blot hybridization analyses were carried out on plant genomic DNA, following treatment with restriction endonucleases as specified in the text. With small-scale preparations of total nucleic acids from individual leaves, plants were analyzed for the presence of AbMV DNA A or DNA B after inoculation of viral genome components. In addition, total nucleic acid preparations served as templates for PCR reactions indicating presence of the transgene. For all applications, nucleic acids were isolated from plant tissues essentially according to Wege and Siegmund (2007), with a slightly modified protocol to enrich plant genomic DNA: 4 g of frozen, ground leaf tissue were extracted by use of 5 ml extraction buffer and an equal total volume of phenol/[chloroform : isoamylalcohol] (1/[25:1]; PCI) as described, the aqueous phase mixed with 1/10 volume 3 M Na acetate pH 4.8, placed on ice, and plant genomic nucleic acids precipitated by slow addition of 0.7 volumes of isopropanol (−20 °C). Following 30 min of incubation on ice, nucleic acids were centrifuged (20 min, 10,000×*g*, 4 °C), washed in 70% ethanol overnight (4 °C), air-dried, and dissolved in 500 µl TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA). After adding 1/10 volume 3 M Na acetate pH 4.8 (4 °C), 2 ml ethanol (−20 °C) were added slowly and precipitated DNA was transferred into 1 ml 70% ethanol (4 °C) by using a glass rod. The mixture was kept at 4 °C overnight, collected by centrifugation (20,000×*g*, 5 min, 4 °C), and air-dried. Nucleic acids were dissolved in 200 µl TE and residual RNA removed by treatment with RNase A (5 µg/ml; 15 min, 37 °C). DNA concentration was determined by gel electrophoresis in the presence of suitable standards and estimated by UV spectroscopy. 30 µg aliquots of genomic DNA were digested with restriction endonucleases following the manufacturer's recommendations, precipitated from the reaction mixtures by use of Na acetate pH 4.8 and an equal volume of isopropanol, resuspended in TE and fragments separated on agarose gels, and transferred onto nylon membranes (GE Healthcare, Hybond NX) after DNA depurination in 0.2 M HCl and neutralization according to standard procedures (Sambrook and Russell, 2001). Alkaline transfer of total nucleic acids (Chomczynski and Qasba, 1984) was used prior hybridization to test for the plants' infection status and was carried out from 1% agarose gels in TBE, after electrophoresis in the presence of 0.5 µg/ml ethidium bromide (EtBr). Hybridization and detection procedures have been described in detail (Wege and Siegmund, 2007) and were the same for Southern blots of genomic DNA and of total nucleic acid preparations and used digoxigenin-labeled DNA probes (see below). Chemiluminescent probe detection via CDP-Star (for genomic DNA analysis) or CSPD (both Roche, Germany) followed the manufacturer's protocols (DIG Application Manual for Filter Hybridization; Roche).

Polymerase chain reaction (PCR) and product purification

PCR was performed to detect AbMV ORFs BC1 and BV1 in genomic DNA present in total nucleic acid preparations of healthy transgenic plants, using 10 µg nucleic acids per 50 µl reaction volume and *Taq* polymerase supplemented with "Q-Solution" (Qiagen, Hilden). Primers and amplification conditions were the same as in Wege and Jeske (1998), annealing ORF BC1 primers at 60 °C, and ORF BV1 primers at 57 °C. DNA fragments used to produce AbMV DNA A- or DNA B-specific hybridization probes without CR sequences were generated by analogous PCR reactions, using primer pairs amplifying either DNA A between start codons of ORFs AC1 and AV1 (annealing at 62 °C), or DNA B between start codons of ORFs BC1 and BV1 (60 °C), respectively. All primers contained 20 nucleotides of the respective ORFs. DNA fragments representing T-DNA sequences close to the LB, or in the *npt II* gene, respectively were amplified from pBIN19 (Bevan, 1984), using primer pair "LB" (DP-T-DNA-LBa: 5′AAC AGC TGA TTG CCC TTC AC3′ and DP-T-DNA-LBb: 5′ATT CAC TGG CCG TCG TTT TA3′, annealing at 57 °C) or "RB" (DP-T-DNA-RBa: 5′CGA AGA ACT CCA GCA TGA GA3′ and DP-T-DNA-RBb: 5′ AAA TGC TCC ACT GAC GTT CC3′, annealing at 57 °C). Prior to probe labeling, PCR fragments were purified by elution from agarose gels by means of GFX kit (GE Healthcare).

Viral nucleic acid in situ localization

Biotin labeling, nonradioactive *in situ* hybridization (ISH), and detection procedures on tissue sections were carried out with a probe detecting both AbMV DNA A and DNA B (total AbMV DNA A excised from plasmid pDE3 (Evans and Jeske, 1993), gel-purified prior to labeling) as described (Morilla et al., 2004). Probes were visualized by a blue precipitate, using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit for horseradish peroxidase (HRP; catalogue no. SK-4400; Vector Laboratories) in combination with streptavidin–HRP conjugate (no. 1089153; Roche), following the procedures specified by Morilla et al. (2004). *N. benthamiana* leaf explants from different transgenic

plants in the three lines analyzed in this study, from wt plants, and from line 14 (non-transgenic control processed via tissue culture) were embedded into Paraplast plus paraffin embedding medium (Oxford, Sherwood Medical, St. Louis, MO, USA) at 42 days post inoculation, according to Zhang et al. (2001). Samples represented all inoculum combinations (mock, DNA B, DNA A, DNA A + B) in all five plant lines, of which sectors from typical leaves up to 20 mm in length, harboring class 2 or 3 veins centrally, were embedded after testing positive for the expected infection status by Southern analysis. In two independent experiments, 10 μm sections (as specified in the Results) were analyzed for AbMV DNA distribution and results documented by bright-field microscopy (Axiophot; Zeiss, adapted to digital camera Canon PowerShot G1). Representative specimens were stained for 5 min in 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in water and fluorescent DNA detected by epifluorescence microscopy, confirming the identity of unlabeled and substrate-covered nuclei (double brightfield and epifluorescence illumination; data not shown).

Hybridization probes

Full-length AbMV-DNA A inserted into pDE3, detecting both AbMV genome components (Evans and Jeske, 1993), was released from the plasmid by digestion with *Pst*I and *Bgl*I, gel-purified (GFX kit, GE Healthcare), biotinylated (Morilla et al., 2004), and used in ISH experiments (as described above). Filter hybridization made use of PCR-derived DNA fragments specific for AbMV DNA A, DNA B, a region close to the T-DNA LB in pBIN19, or the *npt II* gene, respectively, as specified above, labeled with digoxigenin (DIG) by use of DIG-High Prime labeling kit (No. 1 585 606, Roche, Germany) according to the manufacturer's instructions.

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