Virology **290,** 249–260 (2001)
doi:10.1006/viro.2001.1185, available online at http://www.idealibrary.com on IDEAL®

Movement Proteins (BC1 and BV1) of Abutilon Mosaic Geminivirus Are Cotransported in and between Cells of Sink but Not of Source Leaves as Detected by Green Fluorescent Protein Tagging

Shuo Cheng Zhang, Christina Wege, and Holger Jeske[1]

Department of Molecular Biology and Plant Virology, Biological Institute, University of Stuttgart, Pfaffenwaldring 57, D-70550 Stuttgart, Germany

Received May 10, 2001; returned to author for revision August 3, 2001; accepted September 6, 2001

Two movement proteins (BV1 and BC1) facilitate the intra- and intercellular transport of begomoviruses in plants. In contrast to other geminiviruses the movement protein BC1 of *Abutilon mosaic virus* (AbMV) remained in the supernatant after centrifuging plant extracts at 20,000 *g*. To test whether this unusual behavior results from a distinct intracellular distribution of the protein, the *BC1* gene has been fused to the gene of green fluorescent protein (*GFP*). The resulting plasmids were delivered into nonhost plants (*Allium cepa*) as well as into mature and immature cells of host plants (*Nicotiana tabacum, N. benthamiana*) by biolistic bombardment for transient expression *in planta*. BC1 directed GFP to two different cellular sites. In the majority of nonhost cells as well as in mature cells of host leaves, BC1 was mainly localized in small punctate flecks at the cell periphery or, to a lesser extent, around the nucleus. In sink leaves of host plants, GFP:BC1 additionally developed disc-like structures in the cell periphery. Cobombardment of GFP:BC1 with its cognate infectious DNA A and B did not change their subcellular distribution patterns in source leaves but led to the formation of peculiar needle-like structures in sink leaves. The nuclear shuttle protein (BV1) of AbMV accumulated mainly inside the nuclei as shown by immunohistochemical staining and GFP tagging. In sink cells of host plants it was mobilized to the plasma membrane and to the nucleus of the neighboring cell by coexpressed BC1, GFP:BC1, BC1:GFP, or after cobombardment with the cognate viral DNA. Only under these conditions were GFP:BC1 and BC1:GFP also found in the recipient cell.

INTRODUCTION

Viruses move through plants in two steps: cell-to-cell transport, usually via plasmodesmata, and long-distance transport through the phloem (for recent reviews see Ding *et al.,* 1999; Lazarowitz, 1999; Lazarowitz and Beachy, 1999). For cell-to-cell transport, two mechanisms have been discovered for different viruses. One involves the binding of a movement protein to viral RNA and opening of the plasmodesmata (Carrington *et al.,* 1996; Citovsky *et al.,* 1990; Fujiwara *et al.,* 1993; Osman *et al.,* 1992; Wolf *et al.,* 1989). The other is dependent on tubule formation (Kasteel *et al.,* 1997; Perbal *et al.,* 1993; Storms *et al.,* 1995; Wellink *et al.,* 1993).

In contrast to most RNA viruses, the DNA-containing geminiviruses replicate in the nucleus and must exploit additional mechanisms to be transported from the nucleus into the cytoplasm and from one cell to another. Among geminiviruses, the begomoviruses (Rybicki *et al.,* 2000) are characterized by a bipartite genome (DNA A and DNA B), in which DNA B encodes two movement proteins (MPs) called BC1 and BV1 (Etessami *et al.,* 1988; Evans and Jeske, 1993; von Arnim *et al.,* 1993). BC1 mediates cell-to-cell transport, whereas BV1 is responsible for the export of viral DNA from the nucleus. Both proteins cooperate in a way that is currently under debate. Immunological investigations using either transient expression of the two *Squash leaf curl virus* (SqLCV) MPs in protoplasts or systemically infected plants (Ward *et al.,* 1997) support a model in which BV1 exports the viral genomes from the nucleus into the cytoplasm and back to the nucleus as a nuclear shuttle protein, and then BC1 directs the BV1–DNA complexes through modified plasmodesmata, trafficking them from cell to cell (Lazarowitz and Beachy, 1999; Pascal *et al.,* 1994; Sanderfoot *et al.,* 1996; Sanderfoot and Lazarowitz, 1995, 1996). A different model results from investigations on *Bean dwarf mosaic virus* (BDMV). Microinjection experiments delivering bacterially expressed BDMV proteins into mesophyll cells suggested that BV1 shuttled viral DNA from the nucleus to the cytoplasm and that viral DNAs were then transferred from BV1 to BC1 proteins, followed by cell-to-cell movement of the BC1–DNA complexes (Noueiry *et al.,* 1994; Rojas *et al.,* 1998). So far it is not clear whether the two models reflect distinct transport machineries utilized by the two begomoviruses or whether the different experimental methods used resulted in a variable behavior of the viral proteins (Carrington *et al.,* 1996).

Abutilon mosaic virus (AbMV; Wege *et al.,* 2000) is phloem-limited in *Abutilon* (Jeske *et al.,* 1977; Abouzid *et*

[1] To whom correspondence and reprint requests should be addressed. Fax: +49-711-685-5096. E-mail: holger.jeske@po.uni-stuttgart.de.

0042-6822/01 $35.00

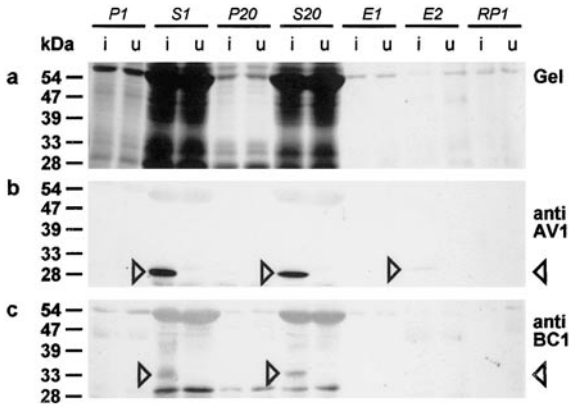

FIG. 1. Gel and Western blot analysis of AbMV BC1 protein from infected plants. Serva-Violet-stained gel (a) and Western blot localization of AbMV proteins (open arrowheads) AV1 (b) or BC1 (c) in pellets (P) or supernatants (S) after differential centrifugation from homogenates of *N. benthamiana*; i: systemically infected, u: uninfected. E: Extracts from P1 using Triton X-100 (E1) or urea/SDS/β-mercaptoethanol, 100°C (E2). RP1: Remainder of extracted P1. Separated samples were from equal amounts of plant material. Molecular weight standards are indicated in kDa.

al., 1988; Horns and Jeske, 1991) as well as in the experimental host *Nicotiana benthamiana* (Wege *et al.,* 2001). *African cassava mosaic virus* (ACMV), a distantly related begomovirus, enters all cell types of the leaf in *N. benthamiana* (Wege *et al.,* 2001). ACMV BC1 was harvested in the so-called "cell wall-enriched fraction" upon differential centrifugation (von Arnim *et al.,* 1993). We will show that AbMV BC1 remains in the supernatant under similar experimental conditions, raising the interesting question of whether movement proteins of geminiviruses with different tissue tropisms accumulate at different sites within the cells.

To this aim, we compared the intracellular distribution of AbMV BC1 in nonhost (*Allium cepa*) and host (*N. benthamiana, N. tabacum*) cells *in vivo* by transient expression of AbMV BC1 fused to the reporter gene of green fluorescent protein (*GFP*). Although AbMV BC1 did not cosediment with the cell wall during subcellular fractionation, it was present close to the cell wall in nonhost and host cells after *in vivo* expression. In a more detailed study (Aberle *et al.,* 2001) AbMV BC1 was localized at the protoplasmic face of the plasma membrane and of vesicles in yeast cells. The functionality of AbMV BC1, even in nonphloem tissues, was shown by its ability to mobilize BV1 to neighboring cells.

RESULTS

Subcellular localization of BC1 in fractionation experiments

Using differential centrifugation on homogenates from systemically infected *N. benthamiana*, von Arnim *et al.* (1993) showed that ACMV BC1 pelleted into the so-called cell wall-enriched fraction after low-speed centrifugation. Similar findings were reported for SqLCV BC1 protein in transgenic plants (Pascal *et al.,* 1993). Following the protocol of von Arnim *et al.* (1993), AbMV BC1 remained completely in the supernatant, as did most of the AbMV coat protein AV1 (Fig. 1). As expected from the low number of AbMV-infected cells (Horns and Jeske, 1991), only a small amount of BC1 protein was detectable in Western blots. The different behavior of AbMV BC1 might result from a lower concentration of the protein in the plant extract, a different localization within the cell, or a distinct property of AbMV BC1 protein with respect to complex formation.

Construction of fusion genes

To discriminate between these possibilities, AbMV *MP* genes were fused to *mGFP4* (Haseloff *et al.,* 1997) or the red-shifted fluorescence variant *smRS-GFP* (Davis and Vierstra, 1998). The fusion genes as well as the unfused AbMV *BC1* or *GFP* genes were cloned between the CaMV 35S promoter and nopaline synthetase (nos) terminator in plant expression vectors, resulting in the plasmids p35S:AbMV BC1, pmGFP4:AbMV BC1, pAbMV BC1:mGFP4, psmRS-GFP:AbMV BC1, and psmRS-GFP:AbMV BV1 (Fig. 2). These constructs were delivered into different tissues by particle gun bombardment and analyzed using epifluorescence or confocal laser scanning microscopy (CLSM).

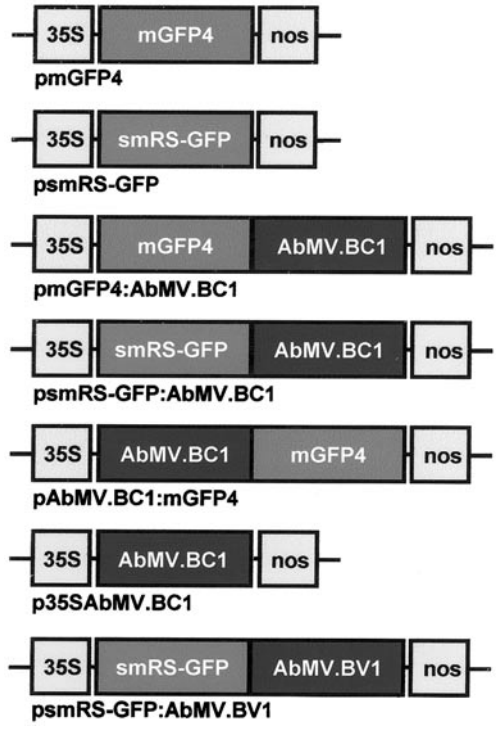

FIG. 2. Gene constructs used in this study. Expression cassettes including the CaMV 35S promoter, either *GFP* gene or fusion constructs, and *NOS* terminator were inserted into a pUC19 plasmid.

Expression of AbMV BC1 in nonhost plant cells

The large, transparent epidermal cells of onion bulb scales are ideal to visualize the fundamental behavior of GFP:BC1 in living cells. Green fluorescence developed in cells transformed with each of the constructs.

N-terminal or C-terminal fusions (mGFP4:AbMV BC1, Figs. 3b, 3c, and 3d, and AbMV BC1:mGFP4, data not shown) behaved identically in preliminary experiments, indicating that the location of GFP in the fusion did not alter the subcellular distribution. In subsequent studies only the former construct was used. Five to six hours postbombardment (hpb), green fluorescence of mGFP4:AbMV BC1 was evenly distributed at the periphery of transformed cells (Fig. 3b). A quarter of the cells retained this distribution without any change for more than 43 hpb (Table 1). In the other cells, the fluorescence intensity increased with incubation time to form fine fluorescent flecks (Fig. 3c). Sixty percent of these cells exhibited these small flecks exclusively at the periphery of the cell. Until 30 hpb, the flecks increased and then remained in this form and size (Table 1). Fifteen percent of the fluorescent cells formed small dotted foci around the nucleus (Fig. 3d), as confirmed by analyzing optical sections of CLSM images and by DNA counterstain with 4′,6-diamidino-2-phenylindole (DAPI) (Figs. 5a–5c).

As already described by Scott *et al.* (1999), unfused mGFP4 or smRS-GFP was found in the nucleus and the cytoplasm, including transvacuolar strands (Fig. 3a).

Expression of AbMV BC1 in mature host plant cells

The same plasmids were delivered into cells of source leaves of *N. tabacum* or *N. benthamiana*. As above, fluorescent cells were visible by 4–5 hpb and remained fluorescent for up to 8 days when the cut leaves were incubated at 24°C in the dark.

smRS-GFP:AbMV BC1 exhibited stronger fluorescence than mGFP4:AbMV BC1, but they were otherwise indistinguishable in both hosts. Therefore, the former was used henceforth. Fusing BC1 to the N- or C-terminus of GFP did not change BC1 behavior.

At 48 hpb, only a few cells showed an even fluorescence close to the cell wall (Fig. 3e), whereas 57% of the fluorescent cells exhibited small flecks at the cell periphery (Fig. 3f) and 23% showed punctate bodies surrounding the nucleus (data not shown, Table 1). In a few cells fluorescence at both locations occurred simultaneously. In general, smRS-GFP:AbMV BC1 was well expressed in epidermis, trichomes, and guard cells of both host plants (data not shown). In conclusion, AbMV BC1 protein had the same principal subcellular behavior both in nonhosts and in hosts, and so we can exclude that host-specific factors are necessary for this basic distribution pattern. During these experiments BC1 was only detected in single cells and never in neighboring cells.

GFP:AbMV BC1 induced disc-like structures in sink leaf cells of host plants

Onion cells and mature *Nicotiana* leaves are helpful to check the constructs for GFP expression and to determine the basic distribution capabilities of the fusion proteins. During natural infection, however, the main sites of virus replication and transport are younger tissues of host leaves that are actively growing.

In contrast to its behavior in source leaves, smRS-GFP:AbMV BC1 appeared as disc-like structures in the cell cortex of sink leaves (Figs. 3h and 3i). The discs were frequently seen as stacks from the side (Fig. 3h, arrowhead). Although unfused GFP spread to neighboring cells (Fig. 3g), GFP:AbMV BC1 never did so.

Distribution of GFP:BC1 coinfected with AbMV DNA A and DNA B

The distribution of BC1 protein might be rearranged if the *BC1* gene is coinoculated with the cognate viral DNA. In mature leaf cells of *N. tabacum* and *N. benthamiana* smRS-GFP:AbMV BC1 distribution was unaltered in the presence of AbMV DNA A and B (data not shown).

In sink leaves of both hosts, however, the fluorescent structures indeed changed. Following a fuzzy appearance of fluorescence at 24 and 76 hpb (data not shown), needle-like structures developed close to the cell periphery in epidermal cells (Figs. 3j–3k), trichomes, and guard cells (data not shown) after 5 dpi. In a few cells, smRS-GFP:AbMV BC1 accumulated around the nucleus as shown before (Table 1).

No transport of GFP:BC1 to adjacent cells was observed under these conditions.

The localization of BC1 in relation to BV1

A rearrangement of BC1 upon cotransformation with the cognate viral DNA might depend on either the presence of genomic length viral DNA or the coexpression of the movement protein BV1. In addition, the viral coat protein (AV1) might have a similar influence as it may exhibit redundant functions with BV1 (Qin *et al.,* 1998).

To analyze the distribution of AV1 and BV1, paraffin-embedded sections of leaves and buds from AbMV-infected *Abutilon sellovianum* and *N. benthamiana* were probed with AV1-, BV1-, and BC1-specific antisera (Wege and Jeske, 1998). AV1 and BV1 were present in a low number of cells and exclusively in phloem tissue as expected from the distribution of viral DNA determined by *in situ* hybridization (Horns and Jeske, 1991; Wege *et al.,* 2001). Analyzing consecutive sections (Figs. 4a–4d) revealed that AV1 and BV1 were coexpressed in the same cell. The nuclear localization of the stain was confirmed by DAPI counterstain (data not shown). No other intracellular AV1- or BV1-specific, nor BC1-specific signals, were detectable under these experimental con-

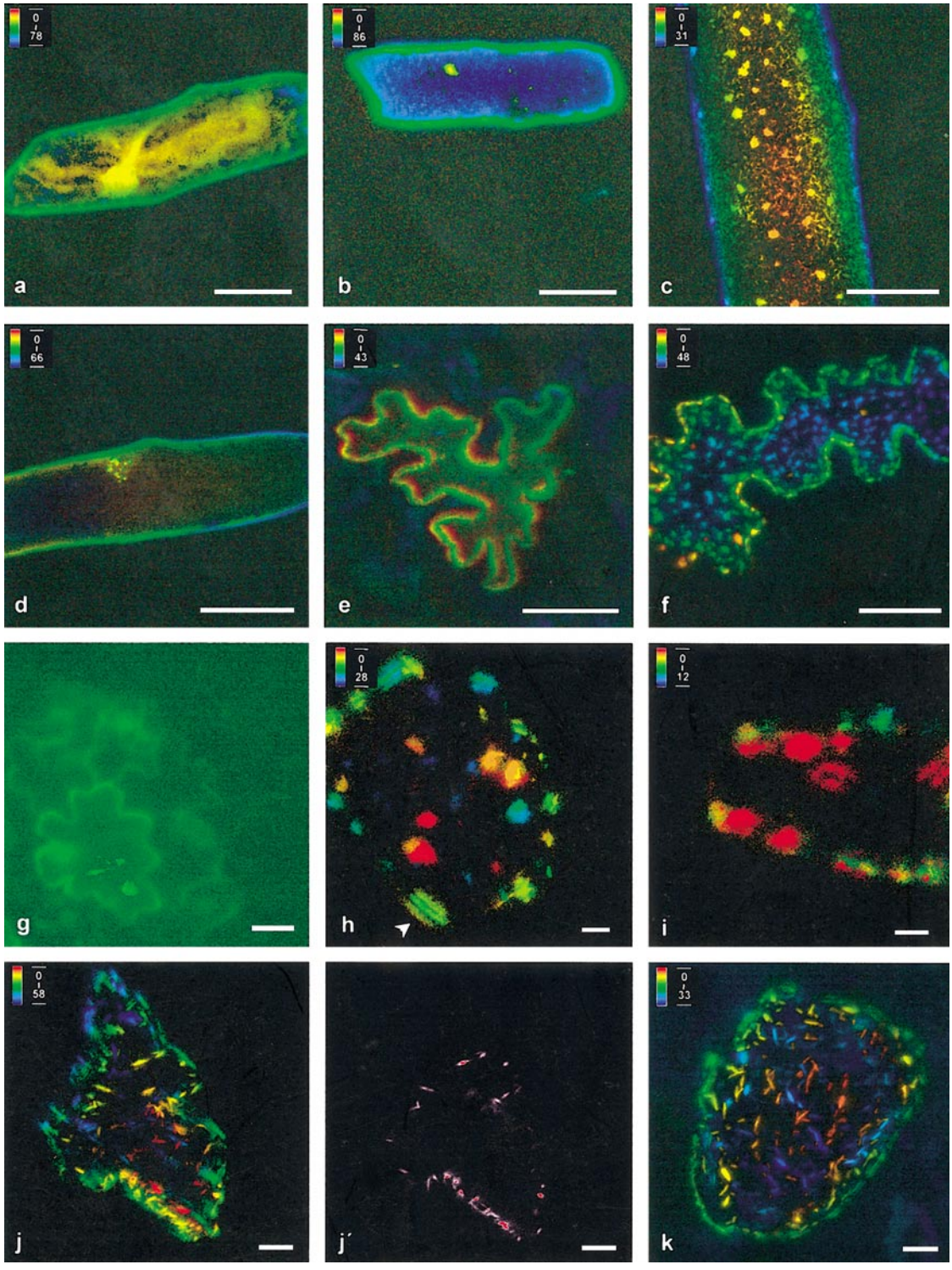

FIG. 3. Patterns of mGFP4, smRS-GFP, mGFP4:AbMV BC1 and smRS-GFP:AbMV BC1 in epidermal cells of onion bulb scales, and of source and sink leaves of *N. tabacum*. Cells are shown as composite pictures of CLSM optical sections. False colors represent green fluorescence in different depths of the optical section as indicated by the color code bar at the top left corners. (a) Unfused mGFP4 in an epidermal onion cell at 24 hpb showing fluorescence in the cytoplasm and nucleus. (b–d) mGFP4:AbMV BC1 in onion cells; (b and c) fluorescent flecks at the cell periphery at 18 hpb (b) and at 84 hpb (c). (d) Fluorescent punctate spots surrounding the nucleus (see Fig. 5) at 83 hpb. (e and f) Distribution patterns of smRS-GFP:AbMV BC1 in epidermal cells of tobacco source leaves at 10 hpb (e) and 70 hpb (f) showing the formation of flecks at the cell periphery. (g) Unfused smRS-GFP in epidermal cells of tobacco at 5 dpi showing brighter fluorescence in the central cell and weaker fluorescence in neighboring cells. (h and i) smRS-GFP:AbMV BC1 in epidermal cells of sink leaves in *N. tabacum* has induced disc-like structures at 76 hpb. Arrowhead indicates stacked discs which were seen throughout several optical sections. (j–k) Distribution of AbMV BC1 after cobombardment with the cognate viral DNAs. Cobombardment of smRS-GFP:AbMV BC1 with AbMV DNA A and B in cells of sink leaves of *N. tabacum* has induced needle-like structures at 5 dpb (j and j′) and at 8 dpb (k). (j′) Single optical section through the center of the cell in j showing the needle-like structures localized in the cell periphery. Bars represent 50 μm (a–d), 25 μm (e–g), and 10 μm (h–k).

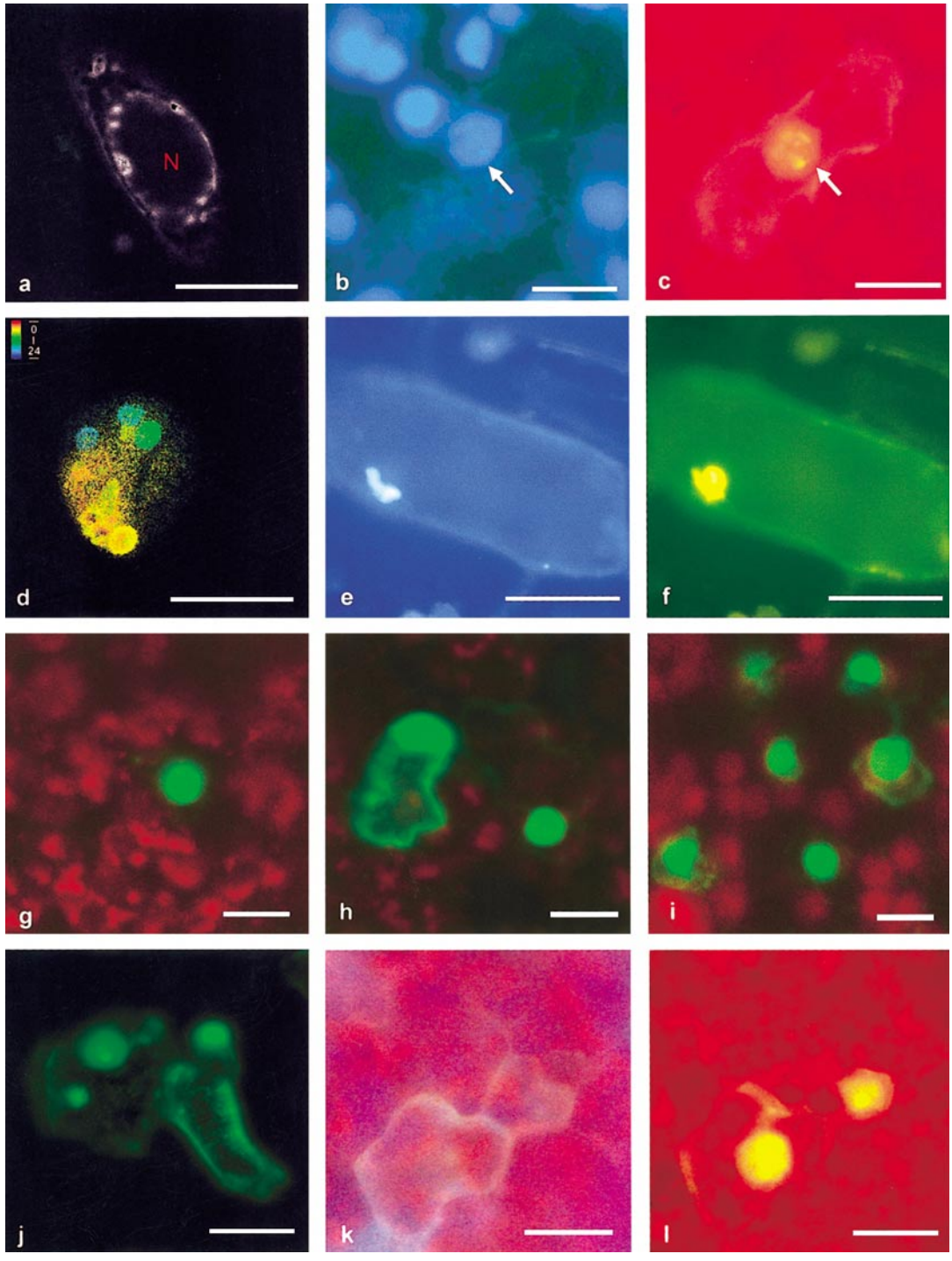

FIG. 5. Distribution of BV1 in relation to BC1 in onion cells (d–f) and sink cells of either *N. tabacum* (g–i) or *N. benthamiana* (a–c, j–l). (a–c) Localization of GFP:BC1 surrounding nuclei. (a) One optical section through a nucleus (N). (b) DAPI fluorescence. Note the nucleus (arrow) and the faint green fluorescence at the periphery allowing the identification of the cell borders. (c) The same cell as in (b) with GFP:BC1 fluorescence; arrow indicates the same nucleus as in (b). (d) One nucleus at higher magnification with smRS-GFP:AbMV BV1 accumulating in globular inclusions in the nucleoplasm at 24 hpb. (e, f) and (k, l) The same cells were photographed with different excitation wavelengths to detect mGFP4:BC1, either alone (e, k) or together with smRS-GFP:BV1 (f, l). smRS-GFP:BV1 was either expressed alone (g), or coexpressed with BC1 (h), AbMV DNA A and B (i), or mGFP4:BC1 (j–l). (b, c, e, f) Epifluorescence micrographs using filter cube I (b, e, k) or II (c, f, l). (a, d, g–j) CLSM. Bars represent 10 μm in a–d, g–l, and 50 μm in e, f.

ditions, suggesting that the amount of BC1 is rather low during natural infection, as expected from Western blot analysis (Fig. 1).

Consistent with these findings, smRS-GFP:AbMV BV1 was predominantly detected in cell nuclei after biolistic plasmid inoculation and transient expression

TABLE 1

Distribution Patterns of Fusion Proteins in *Allium cepa, Nicotiana tabacum,* and *N. benthamiana*

Plasmid construct	Plant	Time (pb)	Cells (total)	Distribution patterns of fluorescence[a]				
				A	B	C	D	E
BC1 alone								
pmGFP4:AbMV BC1	*A. cepa*	43 h	63	16 (25)	38 (60)	9 (15)		
psmRS-GFP:AbMV BC1		45 h	56	11 (20)	39 (70)	6 (10)		
Source leaves:								
psmRS-GFP:AbMV BC1	*N. tabacum*	48 h	44	9 (20)	25 (57)	10 (23)		
psmRS-GFP:AbMV BC1	*N. benthamiana*	60 h	33	3 (9)	27 (82)	3 (9)		
Sink leaves:								
psmRS-GFP:AbMV BC1	*N. tabacum*	78 h	36	9 (25)		4 (11)	23 (64)	
psmRS-GFP:AbMV BC1	*N. benthamiana*	80 h	45	6 (13)		12 (27)	27 (60)	
BC1 and cognate viral DNA A and B								
Source leaves								
psmRS-GFP:AbMV BC1	*N. tabacum*	30 h	42	7 (17)	35 (83)[b]			
		5 d	53	5 (9)	48 (91)[b]			
psmRS-GFP:AbMV BC1	*N. benthamiana*	30 h	38	8 (21)	30 (79)[b]			
		5 d	56	7 (12)	49 (88)[b]			
Sink leaves								
psmRS-GFP:AbMV BC1	*N. tabacum*	2 d	45	6 (13)		11 (24)		28 (62)
		3 d	38	6 (16)				32 (84)
psmRS-GFP:AbMV BC1	*N. benthamiana*	30 h	46	9 (20)		9 (20)		28 (60)
		5 d	67	13 (19)				54 (81)

[a] Number (in parentheses as a percentage) of cells with the respective distribution pattern of fluorescence. (A) evenly distributed fluorescence at cell periphery; (B) punctate flecks at cell periphery; (C) punctate flecks around the nucleus; (D) disc-like structure at cell periphery; (E) needle-like structures at cell periphery.

[b] In these experiments the B and C type were not distinguished.

in nonhost (Figs. 5 d–5f) as well as in host plants (Figs. 5g–5i).

To analyze the relationship between BC1 and BV1, the proteins were fused to two GFPs which can be discriminated by their different excitation wavelengths. When cobombarded into onion cells (Figs. 5e and 5f), BC1 was detected either alone (Fig. 5e) or together with BV1 (Fig. 5f) in the same cell. BV1 was present in the nucleus, whereas BC1 formed a cap around the nucleus. In addition, peripheral fluorescence close to the cell wall was observed during these experiments as shown before. With the chosen constructs it was impossible to determine whether BV1 was present in the peripheral signals together with BC1. Therefore, unfused BC1 was coexpressed with smRS-GFP:AbMV BV1, but the protein did not redirect GFP:BV1 to the cell periphery in onion cells (data not shown).

The behavior of GFP:BV1 was only altered after bombardment of sink cells from host plants (Figs. 5g–5i). In this case, GFP:BV1 stayed in the nucleus when expressed alone (Fig. 5g) but moved to the periphery of the cell and to neighboring cells when coexpressed with BC1 (Fig. 5h) or when coinoculated with AbMV DNA A and B (Fig. 5i). It is improbable that the presence of BV1 in adjacent cells is due to two or more hits during bombardment for the following reasons: First, we rarely detected such hits during all the other experiments (Table 1), only upon coexpression in sink cells. Second, the neighbor cells exhibited a typical feature, namely a strong accumulation of BV1 in the nucleus and only a faint or no signal at the periphery. Third, to provide an impression of the hit frequency after bombardment and to exclude the possibility that two neighboring cells were targeted just by chance, we performed four additional experiments (Table 2) the results of which were consistent with the previous findings. Leaves were bombarded with different construct combinations, and the number of two (paired) or more (>two) adjacent fluorescent cells was compared to the total number of fluorescent cells (Table 2). A low number of paired fluorescent cells (1.3 ± 0.4%) was found if one of the fusion proteins (GFP:BC1 or GFP:BV1) was expressed alone, which may indicate the background probability of hitting two adjacent cells by chance. A significant ($\chi^2 = 173$; $P = <0.0001$) increase in the number of paired cells (7.9 ± 1.5%) was observed if *GFP:BV1* plasmids were cobombarded with *BC1*-containing plasmid constructs (BC1, GFP:BC1, or DNA A and DNA B; Table 2). Moreover, the data underscore the low probability of AbMV proteins to be transported to a neighboring cell, which might be one reason for the limitation of tissue spread during natural infection by AbMV (Wege *et al.,* 2001).

From these results we conclude that GFP:BV1 is mobilized by either BC1 or the viral DNA gene products, not

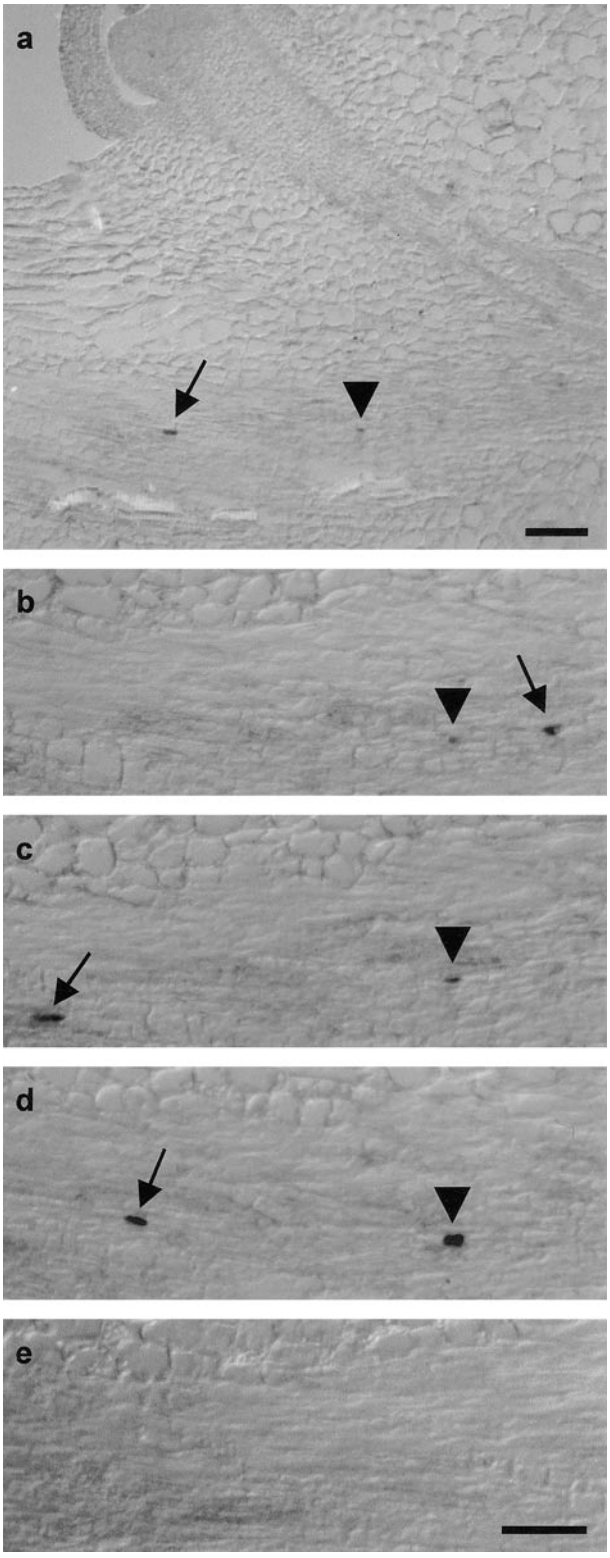

FIG. 4. Immunological detection of AbMV proteins AV1 and BV1 in nuclei. Consecutive sections from a systemically infected *Abutilon sellovianum* stem node with axillary bud ((a) overview; (b–e) magnified consecutive sections; filled arrowheads indicate the same nucleus)). Proteins were detected using AV1 (a, c, d) and BV1 (b) antiserum, or preimmune serum control (preBV1) (e). Arrows and arrowheads indicate AV1- or BV1-specific violet stain precipitated from NBT/BCIP by alkaline phosphatase. Nuclear localization of the violet signals was confirmed by DAPI counterstain (not shown). Note that AV1 and BV1 were present in the same nucleus in consecutive sections (a, b, and c arrowheads). The concentration of BC1 was too low to be detected under these conditions. Bars represent 50 μm ((b-e) at the same magnification)).

only to the cell periphery, but also to the next cell where it is released and transported to the nucleus. This process is even more prominent after cobombardment with DNA A and B (Fig. 5i; Table 2), probably because BC1 can also be expressed in the neighboring cells under these conditions. Under the chosen conditions, we never detected more than two adjacent fluorescent cells if the viral proteins were ectopically expressed from plasmids, but this occurred reproducibly, although again with a low frequency, if full-length viral DNA A and B were cobombarded (Table 2; >two).

To test whether the GFP moiety in BC1 constructs hinders their function, rsGFP:BV1 was coexpressed with either BC1, GFP:BC1, or BC1:GFP in *N. benthamiana* sink leaves. Under these conditions BV1 was mobilized to neighboring cells (Figs. 5j and 5i) and no statistically significant difference between the effects of the BC1 constructs with or without GFP fusion was detected (Table 2; 7.3 $\pm$ 1.5% for BC1 and 7.4 $\pm$ 1.4% for GFP:BC1). Moreover, GFP:BC1 (Fig. 5k) and BC1:GFP (data not shown) also appeared in the adjacent cells. Therefore, we conclude that the GFP fusion is not the reason for the lack of BC1 movement in all of the other experiments. On the contrary, we suggest that complex formation of BV1 and BC1 triggers the transport of both proteins as far as they are expressed in certain competent cells.

DISCUSSION

GFP has been used as a reporter to trace the subcellular and intercellular behavior of the MPs of a bipartite geminivirus in nonhost and host plants. In addition, we have compared the distribution of MPs in source and sink cells of host plants. In contrast to the injection of bacterially expressed proteins, biolistic delivery of *GFP:MP* fusion gene constructs promises a better folding of the encoded proteins and increases the chance of obtaining a physiologically significant posttranslational modification pattern (Itaya *et al.,* 1997). In particular, phosphorylation might play a crucial role in the regulation of viral movement (Sanderfoot and Lazarowitz, 1996; von Arnim *et al.,* 1993; Wege and Jeske, 1998).

The results presented here show that the AbMV BC1 accumulated mainly as punctate spots at the cell cortex, similar to the immunolocalized SqLCV BC1 (Sanderfoot and Lazarowitz, 1995, 1996; Sanderfoot *et al.,* 1996), and also close to the nucleus in certain cells. Nevertheless, AbMV BC1 remained soluble after low-speed centrifugation, in the supernatant which contains free proteins as

TABLE 2

Quantification of Transformation Efficiencies

Treatment	Expt. No.	Fluorescent cells			Percentage	
		Total	Paired	>Two	Paired	>Two
GFP:BC1	1	525	8	0	1.5	0.0
	2	459	7	0	1.5	0.0
	3	507	9	0	1.8	0.0
	4	392	4	0	1.0	0.0
GFP:BV1	1	465	3	0	0.6	0.0
	2	330	3	0	0.9	0.0
	3	123	2	0	1.6	0.0
	4	396	5	0	1.3	0.0
BC1 + GFP:BV1	1	425	41	0	9.6	0.0
	2	371	20	0	5.4	0.0
	3	684	49	0	7.2	0.0
	4	760	53	0	7.0	0.0
GFP:BC1 + GFP:BV1	1	253	14	0	5.5	0.0
	2	382	33	0	8.6	0.0
	3	419	31	0	7.4	0.0
	4	277	22	0	7.9	0.0
DNA A + DNA B + GFP:BV1	1	225	21	2	9.3	0.9
	2	463	39	3	8.4	0.6
	3	624	57	6	9.1	1.0
	4	659	62	8	9.4	1.2

well as microsomes, including plasma membrane vesicles (Nagahashi, 1985).

The basic site of accumulation of AbMV BC1, whether it is close to the cell periphery or the nucleus, was similar in host and nonhost cells, suggesting that host-specific factors are not involved in this fundamental process. Interestingly, we found a plasma membrane localization of BC1 even in fission yeast cells (Aberle *et al.,* 2001). Apart from this general feature, the pattern of BC1 accumulation within the cells depended considerably on the developmental stage of the plant in the case of AbMV. GFP:BC1 induced stacked disc-like structures in epidermal, trichome, and guard cells of sink leaves (Fig. 3). This feature was completely absent from cells of source leaves. Ward *et al.* (1997) provided evidence that BC1-containing tubules might be derived from endoplasmic reticulum. Localization of BC1 protein close to the nucleus as a second main site of accumulation has not been reported so far for other geminiviruses. Very rarely, cells with both sites of accumulation were found, and we assume that the cell physiology governs the distribution of BC1. The change of sites might be regulated by post-translational modifications. Phosphorylation has been shown for BC1 proteins (von Arnim *et al.,* 1993; Pascal *et al.,* 1994; Wege and Jeske, 1998). Wege and Jeske (1998) showed that AbMV BC1 was phosphorylated in *Escherichia coli*, and the pattern of phosphorylation might be different in eukaryotes, thus leading to different targeting of the proteins. Moreover, phosphorylation might be developmentally regulated, explaining the divergent distribution patterns in sink compared to source cells.

In all experiments, whether in host or nonhost plants, in source or sink cells, AbMV GFP:BC1 or BC1:GFP never moved to the neighboring cells if expressed alone. Even cobombardment with DNA A and B did not mobilize these proteins in mature cells. This behavior contrasts to that of unfused GFP which diffused to the neighboring sink cells (Fig. 3g) as has been reported by Oparka *et al.* (1999). Presumably the fusion proteins are too large for passive transport through plasmodesmata. Fluorescently labeled, unfused BDMV BC1 was found in neighboring cells after microinjection (Noueiry *et al.,* 1994; Rojas *et al.,* 1998).

The mobilization of GFP:BC1 and GFP:BV1 exclusively in cells of sink leaves in host plants suggests that GFP does not greatly hinder BC1 and BV1 in this process. BV1 proteins of SqLCV and *Tomato leaf curl virus* (ToLCV) were localized exclusively to the nuclei in infected plants (Pascal *et al.,* 1994) and in transfected protoplasts (Padidam *et al.,* 1999) as confirmed here for AbMV. It has been proposed that BV1 accumulates in nuclei because nuclear import is faster than export (Lazarowitz and Beachy, 1999; Gerace, 1995). In protoplasts, SqLCV BV1 was mobilized to the cell periphery after coexpression with the cognate BC1 (Sanderfoot and Lazarowitz, 1995, 1996; Sanderfoot *et al.,* 1996). Here we show for the first time that BV1 protein is additionally transported to the nucleus of an adjacent cell if coexpressed with BC1.

Evidence from BDMV experiments indicated that both BC1 and BV1 are nucleic acid-binding proteins and recognize ssDNA and dsDNA in a form- and size-specific manner (Noueiry *et al.,* 1994; Rojas *et al.,* 1998). Two models of the interaction of viral DNA with the movement proteins have been discussed. BV1 might carry viral DNA into the cytoplasm where it is displaced by BC1, which then transports the nucleic acid through the plasmodesmata (Noueiry *et al.,* 1994; Carrington *et al.,* 1996). Accordingly, BC1 was shown to mediate cell-to-cell movement of fluorescently labeled ssDNA and dsDNA in microinjection studies. In contrast, BV1 was only able to export injected DNA from the nucleus (Noueiry *et al.,* 1994; Rojas *et al.,* 1998).

In the case of SqLCV, BC1 directed BV1 from the nucleus to the cell periphery, suggesting that BC1 interacts with BV1 *in vivo*. SqLCV BC1 did not bind either ssDNA or dsDNA *in vitro*, whereas BV1 bound ssDNA. Based on this evidence, a movement model has been proposed in which BC1 mediates the transport of BV1–ssDNA complexes via tubules transiently induced by virus infection in developing phloem tissues (Pascal *et al.,* 1994; Ward *et al.,* 1997; Lazarowitz and Beachy, 1999). The apparent discrepancy between the two models has been explained by phloem-limitation of SqLCV, whereas BDMV can also infect nonvascular tissues (Hoefert, 1987; Wang *et al.,* 1996; Lazarowitz and Beachy, 1999).

The observation of AbMV BC1 protein accumulating at the outer surface of the nucleus is a new observation and presumably allows further detail of the process to be visualized. Based on this observation and the detection of BV1 movement to adjacent cells, we propose that BC1 might fetch complexes of viral DNA and BV1 from the outer side of the nuclear envelope and transfer them to plasmodesmata and then to the adjacent cell where the BV1–DNA complex is released for the subsequent nuclear import. As an extension to previous models of geminiviral transport, it seems that, at least for AbMV, the trigger for cell-to-cell transport of BC1 as well as BV1 needs the interaction of both proteins. Moreover, cell-to-cell transport occurs only in certain competent cells of sink leaves. Here we have shown a limited transport of GFP:BV1 and GFP:BC1 to adjacent (mostly) epidermal cells which are not normally infected by AbMV. The significance of these observations for the phloem-limited viral infection has to be established, but the low frequency of cell-to-cell movement of GFP:BV1 might contribute to an explanation for the low percentage of AbMV-infected cells in a variety of hosts (Wege *et al.,* 2001). On the other hand, although AbMV BC1 and/or BV1 may be less efficient for transport than the corresponding proteins of other geminiviruses, the results show that the AbMV proteins still possess, in principle, the ability to move from cell to cell in nonphloem cells.

MATERIALS AND METHODS

Subcellular fractionation

Leaf and upper stem tissue from fully symptomatic, AbMV-infected, or from uninfected *N. benthamiana* were used for differential centrifugation analyses according to von Arnim *et al.* (1993). The tissue was homogenized in liquid nitrogen, and protein was extracted in 2-ml grinding buffer (GB; von Arnim *et al.,* 1993) per gram starting material. The homogenate was centrifuged for 10 min at 1000 *g* using a swing-out rotor to yield pellet P1 and supernatant S1. The latter was centrifuged at 20,000 *g* for 20 min to produce pellet P20 and supernatant S20. Pellet P1 was washed with GB and resuspended in 1–2 ml GB/Triton (1% Triton X-100 in GB) per gram of starting material. Following centrifugation at 1000 *g* for 10 min, the supernatant was removed and named extract E1; the residual pellet washed once in GB/Triton, resuspended in 1–2 ml ESB per gram starting material (von Arnim *et al.,* 1993), boiled for 5 min, and centrifuged again to produce the supernatant extract E2 and its corresponding pellet RP1 (remainder P1). For Western blot analyses, pellets P1, P20, and RP1 were suspended in GB and suitable aliquots of these suspensions as well as of the different supernatants were mixed with equal amounts of 2× SDS sample buffer (Sambrook *et al.,* 1989), boiled for 10 min, centrifuged at 20,000 *g* for 1 min, and run on discontinuous SDS polyacrylamide gels (12.5% polyacrylamide; Laemmli, 1970). The samples separated in either lane were derived from equal amounts of plant material. Protein molecular weight standards were from Pharmacia or Serva. Gels were either fixed and stained with Serva-violet 17 (Serva), or proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, PROTRAN 0.45 μm) by Western blotting (Towbin *et al.,* 1979; Trans-Blot SD, Bio-Rad, Hercules, CA). Air-dried membranes were stained for 5 min in 0.2% Ponceau S in 3% acetic acid and destained in water.

Immunodetection procedures were modified from de Maio (1994) and Harlow and Lane (1988). Following a 1 h blocking step in 3% BSA in PBS pH 7.2 at room temperature, membranes were agitated for 2 × 5 min in PBS-T (0.1% Tween 20 (Sigma) in PBS pH 7.2), 90 min in primary antibody solution (polyclonal antiAbMV antisera or preimmune sera diluted 1:1000 in 2.5% BSA, 0.02% NaN_3 in PBS), 5 min in 1% NP-40 in PBS, 4 × 5 min in PBS-T, 60 min in secondary antibody solution (goat anti-mouse immunoglobulin G, IgG-alkaline phosphatase conjugate, Biotrend) diluted 1:2000 in AP-dilution buffer (1% BSA in 100 mM Tris–Cl pH 7.5, 150 mM $MgCl_2$), 6 × 5 min in TBS-T (0.1% Tween 20 in 25 mM Tris–Cl pH 7.4, 150 mM NaCl), and 5 min in alkaline AP buffer (100 mM Tris–Cl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). Blots were transferred to detection solution (alkaline AP buffer containing 300 μg/ml NBT (Gibco-BRL) and 165 μg/ml BCIP (Gibco-BRL)) and stain precipitation was stopped after 5–15 min by several rinses in 0.5 mM EDTA.

Construction of translational fusion genes and molecular cloning

Standard molecular cloning procedures were applied according to Sambrook *et al.* (1989).

GFP genes. mGFP4 was the version of GFP (Chalfie *et al.,* 1994) according to Haseloff *et al.* (1997), smRS-GFP on plasmid pCD-327 according to Davis and Vierstra (1998), kindly provided by the authors. The plasmids contain the genes flanked by the CaMV 35S promoter and nos terminator inserted into pBIN19 and pUC119, respectively. mGFP4 and smRS-GFP can be distinguished by their excitation wavelength during fluorescence microscopy (see below).

Viral fusion constructs. An *Eco*RI–*Hin*dIII fragment from pBIN35S-mGFP4 (Haseloff *et al.,* 1997) was inserted between the corresponding restriction sites of pUC19, giving rise to pmGFP4. pmGFP4 was first digested with *Eco*RI, filled in with Klenow enzyme, and religated to remove the *Eco*RI site resulting in pmGFP4Δ*Eco*RI. This plasmid was amplified with primers 1 and 2 (Table 3). The PCR product was digested with *Sst*I and religated resulting in pmGFP4(E-S-X). To fuse *mGFP4* with AbMV *BC1*, it was amplified from plasmid AbB (Frischmuth *et al.,* 1990) with primers 3 and 4. The PCR fragment was

TABLE 3

Primers Used in Gene Construction

No.	Name	Sequence (5′ to 3′)[a]	Remarks
1	NOS-*Sst*I-*Xho*I-FOR	TAAGAGCTCGAGTTTCCCCGATCGTTCAAACATTTG	5′ end of nos. terminator + *Sst*I and *Xho*I
2	mGFP4-*Sst*I-*Eco*RI-REV	GCAGAGCTCGAATTCATCCATGCCATGTGTAATCCC	3′ end of gfp + *Sst*I and *Eco*RI
3	AbMVB-*Eco*RI-BC1-FOR	CTTGAATTCATGGATTCTCAGTTAGTAAAT	5′ end of AbMV *BC1* + *Eco*RI
4	AbMVB-*Xho*I-BC1-REV	AATCTCGAGTTATTTCAATGATTTGGCTTG	3′ end of AbMV *BC1* + *Xho*I
5	AbBV1-*Eco*RI-FOR	CCCTTGAATTCATGTACCCGTCTAGGAATAAACG	5′ end of AbMV *BV1* + *Eco*RI
6	AbBV1-*Xho*I-REV	CCCTTCTCGAGTTAACCAATATAGTCAAGGTC	3′ end of AbMV *BV1* + *Xho*I
7	FOR-AbBC1	CAAACGGATCCAACAATGGATTCTCAGTTAGTAAATCC	5′ end of AbMV *BC1* + *Bam*HI
8	REV-AbBC1-GFP	GTTCTTCTCCTTTACTCATTTTCAATGATTTGGCTTG	3′ end of AbMV *BC1* and 5′ end of mgfp4; complementary to No. 9
9	FOR-GFP-AbBC1	CAAGCCAAATCATTGAAAATGAGTAAAGGAGAAC	3′ end of AbMV *BC1* and 5′ end of mgfp4
10	REV-GFP-*Eco*RI	CGGCCAGTGAATTCCCGATCTAGTAACA	Sequence of pUC19 downstream of gfp

[a] Added recognition sites for restriction enzymes are underlined.

digested with *Eco*RI and *Xho*I and inserted into pmGFP4(E-S-X), resulting in pmGFP4:AbMV BC1.

A *Bam*HI–*Nsp*V fragment from psmRS-GFP, containing the *smRS-GFP* gene (Davis and Vierstra, 1998), was exchanged with that of pmGFP4(E-S-X), giving rise to psmRS-GFP(E-S-X). The PCR fragment of the AbMV *BV1* gene, amplified from plasmid AbB (Frischmuth *et al.,* 1990) with primer 5 and 6, was digested with *Eco*RI and *Xho*I and inserted into psmRS-GFP(E-S-X), resulting in psmRS-GFP:AbMV BV1. An *Eco*RI–*Xho*I fragment of AbMV *BC1*, cut from pmGFP4:AbMV BC1, was also inserted into psmRS-GFP(E-S-X), resulting in psmRS-GFP: AbMV BC1.

For 5′-terminal fusion of *GFP* with AbMV *BC1*, the construct was produced by two PCR steps according to Higuchi *et al.* (1988) as modified by Wurch *et al.* (1998). Primers 7 and 8 were used to amplify the AbMV *BC1* gene from plasmid AbB. Primers 9 and 10 were used to amplify *mGFP4* and the nos-terminator from pmGFP4. The amplified AbMV *BC1* and *mGFP4* fragments were fused in frame using an extension polymerization. The resulting fragment was amplified with primers 7 and 10 by a second PCR. It was digested with *Bam*HI and *Eco*RI and exchanged for *GFP* in pmGFP4, resulting in pAbMV BC1:mGFP4.

E. coli (strain DH5α or JM83) was transformed with the resulting plasmids. Purified plasmids from isolated colonies were digested with suitable restriction enzymes to check the size of inserts. The constructs were sequenced with semiautomatic sequencing (Li-Cor DNA-Sequencer 4000L; MWG, Germany). Correct plasmids were amplified in *E. coli* and purified for biolistic transient transformation using NUCLEO-BOND cartridges AX 500 (Macherey-Nagel, Germany).

Biolistic inoculation of plant tissues

Sixty milligrams of 1.0 μm gold particles (Bio-Rad) were washed twice with 70% ethanol, once in sterile water with moderate vortexing for 10 min and suspended in 1 ml 50% (v/v) glycerol. Five microliters of plasmid DNA (1 μg/μl) harboring either *GFP* alone as a control or fusion constructs were mixed with 25 μl gold suspension by vortexing for 3 min and kept on ice for 10 min. Ten microliters 0.1 M spermidine and 25 μl 2.5 M $CaCl_2$ were added and vortexed for 4 min. DNA-coated gold particles were collected by a brief centrifugation, washed twice with 70% ethanol, and resuspended in 36 μl 98% ethanol. Six microliters of the suspension was spread on each plastic carrier disc.

Onion bulb scales were cut into pieces of 1.5 $\times$ 1.5 cm and kept in Petri dishes. Completely expanded and small young sink leaves (*N. tabacum* Samsun nn or *N. benthamiana*) were cut from 6- to 8-week-old plants which were grown in the glasshouse and laid immediately with the lower side up on a sterile wet Whatman filter in a plastic Petri dish. The freshly cut ridges were covered with small pieces of wet napkin paper. Sink or source status of leaves was determined by monitoring in parallel the translocation of 5(6)-carboxyfluorescein diacetate (CF) according to Roberts *et al.* (1997).

Plasmid DNA was delivered into the lower side of leaves or into the inner peels of bulb scales using a particle gun (PDS-1000/He; Bio-Rad) using either 900 psi rupture discs in the case of leaves (Marc *et al.,* 1998) or 1100 psi rupture discs in the case of bulb scales under a vacuum of 25 in. Hg. For cobombardment of different constructs the plasmids (encoding BC1, BV1, or DNA A and DNA B) were mixed before gold-coating to deliver them into the same cell with similar efficiency. After bombardment, explants were kept in the dark at 24 or 4°C.

Fluorescence microscopy.

For routine observation an Axioskop (Zeiss Corp., Germany) was used including filter cube I (365-nm excitation; 395-nm beam splitter; 429-nm long-pass emission) for mGFP4 fluorescence and filter cube II (450- to 490-nm excitation; 510-nm beam splitter; 520-nm band-pass

emission) for smRS-GFP. Photographs were taken using an MC-100 Spot-Camera (Zeiss Corp.) and Fujichrome Provia 400 daylight positive film.

Confocal laser scanning microscopy and image processing

Confocal laser scanning microscopy was done using a LSM model 410 invert (Zeiss Corp.). Fluorescent cells were imaged using a 488-nm argon laser, 670- to 810-nm band filter for red fluorescence of chloroplasts and a 510- to 540-nm emission filter for GFP. Optical sections at 0.5- to 2-μm intervals were made, attenuating the laser intensity to the lowest possible to reduce photobleaching of GFP.

The collected optical sections were digitally processed and assembled by use of the manufacturer's software (Carl Zeiss LSM Program, Germany). The green fluorescence was transformed to false colors to indicate the depth of the optical sections, resulting in pseudo-three-dimensional pictures.

Immunolocalization of AbMV BV1 in paraffin sections

Leaf segments and axillary bud explants from *A. sellovianum* were fixed at room temperature for 1 h in 4% formaldehyde, freshly prepared from paraformaldehyde, in MTSB (microtubule stabilizing buffer: 50 mM PIPES pH 6.9 with KOH, 5 mM EGTA, 5 mM $MgSO_4$), and 8 to 14 h at 10°C in 6% formaldehyde in 0.15 M sodium phosphate buffer pH 7.6. Specimens were agitated at room temperature for 2 × 15 min in phosphate buffer, dehydrated in a graded series of ethanol, and infiltrated with Histo-Clear (National Diagnostics):ethanol 1:1 for 1 h, with Histo-Clear 3 × 1 h at room temperature and with Histo-Clear:Paraplast Xtra (Oxford Labware, Sherwood Medical) overnight at 42°C. Infiltration with pure Paraplast Xtra was performed at 58°C for 3 days, replacing the wax once per day. Specimens were solidified on ice, sections (5–10 μm) were collected on silane-coated slides (bind-silane GF31, Wacker), and paraffin was removed by extraction with Roticlear (Roth), isopropanol, and ethanol.

Immunodetection procedures were carried out at room temperature. Sections were rehydrated 2 × 5 min in PBS, incubated for 15 min in 50 mM glycine in PBS, 2 × 5 min in PBS, 10 min in blocking solution A (3% BSA, 5% normal goat serum (Jackson ImmunoResearch) in PBS), 10 min in blocking solution B (0.2% gelatin (Merck), 0.5% BSA in PBS), 2 × 3 min in PBS-T and 2.5 h in primary antibody solution (polyclonal mouse antiserum or preimmune serum (Wege and Jeske, 1998), diluted 1:3000 in 3% BSA in PBS). Following 3 × 7 min washes with 1% NP-40 (Fluka) in PBS and 2 × 10 min in TBS-T, sections were subjected to secondary antibody solution (goat anti-mouse immunoglobulin G, H&L, IgG-alkaline phosphatase conjugate (Biotrend), diluted 1:250 in AP-dilution buffer (1% BSA in 100 mM Tris–HCl pH 7.5, 150 mM $MgCl_2$) for 1 h). The slides were washed 4 × 5 min in TBS-T, 2 × 5 min in detection buffer 1 (0.1 M maleic acid, 0.15 M NaCl pH 7.5), and 5 min in alkaline AP buffer. The latter was replaced by detection solution (see above); stain development was observed microscopically and stopped after 5 to 10 min by rinsing in 0.5 mM EDTA. For visualization of DNA, sections were counterstained with DAPI (1 μg/ml in water) for 5 min and mounted with Citifluor (Plano). Specimens were analyzed with Zeiss-Axioskop epifluorescence microscopes using differential interference contrast (DIC) equipment to enhance contrast. Pictures were taken on Kodak Ektachrome 64T films with Zeiss-Axiophot photo equipment.

ACKNOWLEDGMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Je 116/5-3). We thank Prof. Dr. R. Ghosh, PD Dr. T. Frischmuth, and Dr. B. Ding for helpful discussions, Dr. Haseloff for pmGFP4, Drs. Davis and Vierstra for pCD-327, Prof. Dr. H.-D. Görtz for providing the CLSM facilities, and Dr. J. Brenner and Dr. M. Schweikert for help with the laser scanning microscopy.

REFERENCES

Aberle, H.-J., Rütz, M.-L., Karayavuz, M., Frischmuth, S., Wege, C., Hülser, D., and Jeske, H. (2001). Localizing the movement proteins of Abutilon mosaic geminivirus in yeast by subcellular fractionation and freeze fracture immuno-labeling. *Arch. Virology*, in press.

Abouzid, A. M., Barth, A., and Jeske, H. (1988). Immunogold labeling of the Abutilon mosaic virus in ultrathin sections of epoxy resin embedded leaf tissue. *J. Ultrastruct. Res.* **99,** 39–47.

Carrington, J. C., Kasschau, K. D., Mahajan, S. K., and Schaad, M. C. (1996). Cell-to-cell and long-distance transport of viruses in plants. *Plant Cell* **8,** 1669–1681.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263,** 802–805.

Citovsky, V., Knorr, D., Schuster, G., and Zambryski, P. (1990). The P30 movement protein of tobacco mosaic virus is a single-strand nucleic acid binding protein. *Cell* **60,** 637–647.

Davis, S. J., and Vierstra, R. D. (1998). Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. *Plant Mol. Biol.* **32,** 521–528.

de Maio, A. (1994). Protein blotting and immunoblotting using nitrocellulose membranes. *In* "Protein Blotting. A Practical Approach" (Dunbar, B. S., Ed.), pp. 11–32. IRL Press, New York.

Ding, B., Itaya, A., and Woo, Y.-M. (1999). Plasmodesmata and cell-to-cell communication in plants. *Int. Rev. Cytol.* **190,** 251–316.

Etessami, P., Callis, R., Ellwood, S., and Stanley, J. (1988). Delimitation of essential genes of cassava latent virus DNA 2. *Nucleic Acids Res.* **16,** 4811–4829.

Evans, D., and Jeske, H. (1993). DNA B facilitates, but is not essential for, the spread of Abutilon mosaic virus in agroinoculated *Nicotiana benthamiana*. *Virology* **194,** 752–757.

Frischmuth, T., Zimmat, G., and Jeske, H. (1990). The nucleotide sequence of abutilon mosaic virus reveals prokaryotic as well as eukaryotic features. *Virology* **178,** 461–468.

Fujiwara, T., Giesman-Cookmeyer, D., Ding, B., Lommel, S. A., and Lucas, W. J. (1993). Cell-to-cell trafficking of macromolecules through plasmodesmata potentiated by the red clover necrotic mosaic virus movement protein. *Plant Cell* **5,** 1783–1794.

Gerace, L. (1995). Nuclear export signals and the fast track to the cytoplasm. *Cell* **82,** 341–344.

Harlow, E., and Lane, D. (1988). "Antibodies." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Haseloff, J., Siemering, K. R., Prasher, D. C., and Hodge, S. (1997). Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. *Proc. Natl. Acad. Sci. USA* **94,** 2122–2127.

Higuchi, R., Krummel, B., and Saiki, R. K. (1988). A general method of in vitro preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions. *Nucleic Acids Res.* **16,** 7351–7367.

Hoefert, L. L. (1987). Association of Squash leaf curl virus with nuclei of squash vascular cells. *Phytopathology* **77,** 1596–1600.

Horns, T., and Jeske, H. (1991). Localization of Abutilon mosaic virus DNA within leaf tissue by *in situ* hybridization. *Virology* **181,** 580–588.

Itaya, A., Hickman, H., Bao, Y., Nelson, R. S., and, Ding, B. (1997). Cell-to-cell trafficking of cucumber mosaic virus movement protein: Green fluorescent protein fusion produced by biolistic gene bombardment in tobacco. *Plant J.* **12**, 1223–1230.

Jeske, H., Menzel, D., and Werz, G. (1977). Electron microscopic studies on intranuclear virus-like inclusions in mosaic-diseased Abutilon sellowianum Reg. *Phytopath. Z.* **89,** 289–295.

Kasteel, D. T. J., van der Wel, N. N., Jansen, K. A. J., Goldbach, R. W., and van Lent, J. W. M. (1997). Tubule-forming capacity of the movement proteins of alfalfa mosaic virus and brome mosaic virus. *J. Gen. Virol.* **78,** 2089–2093.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lazarowitz, S. G. (1999). Probing plant cell structure and function with viral movement proteins. *Curr. Opin. Plant Biol.* **2,** 332–338.

Lazarowitz, S. G., and Beachy, R. N. (1999). Viral movement proteins as probes for intracellular and intercellular trafficking in plants. *Plant Cell* **11,** 535–548.

Marc, J., Granger, C. L., Brincat, J., Fisher, D. D., Kao, T., McCubbin, A. G., and Cyr, R. J. (1998). A GFP-MAP4 reporter gene for visualizing cortical microtubule rearrangements in living epidermal cells. *Plant Cell* **10,** 1927–1940.

Nagahashi, G. (1985). The marker concept in cell fractionation. *In* "Modern Methods of Plant Analysis: Cell Components" (Linskens, H. F., and Jackson, J. F., Eds.), pp. 66–84. Springer-Verlag, Berlin/Heidelberg.

Noueiry, A. O., Lucas, W. J., and Gilbertson, R. L. (1994). Two proteins of a plant DNA virus coordinate nuclear and plasmodesmatal transport. *Cell* **76,** 925–932.

Oparka, K. J., Roberts, A. G., Boevink, P., Santa Cruz, S., Roberts, I., Pradel, K. S., Imlau, A., Kotlizky, G., Sauer, N., and Epel, B. (1999). Simple, but not branched, plasmodesmata allow the nonspecific trafficking of proteins in developing tobacco leaves. *Cell* **97,** 743–754.

Osman, T. A., Hayes, R. J., and Buck, K. W. (1992). Cooperative binding of the red clover necrotic mosaic virus movement protein to single-stranded nucleic acids. *J. Gen. Virol.* **73,** 223–227.

Padidam, M., Beachy, R. N., and Fauquet, C. M. (1999). A phage single-stranded DNA (ssDNA) binding protein complements ssDNA accumulation of a geminivirus and interferes with viral movement. *J. Virol.* **73,** 1609–1616.

Pascal, E., Goodlove, P. E., Wu, L. C., and Lazarowitz, S. G. (1993). Transgenic tobacco plants expressing the geminivirus BL1 protein exhibit symptoms of viral disease. *Plant Cell* **5,** 795–807.

Pascal, E., Sanderfoot, A. A., Ward, B. M., Medville, R., Turgeon, R., and Lazarowitz, S. G. (1994). The geminivirus BR1 movement protein binds single-stranded DNA and localizes to the cell nucleus. *Plant Cell* **6,** 995–1006.

Perbal, M. C., Thomas, C. L., and Maule, A. J. (1993). Cauliflower mosaic virus gene I product (P1) forms tubular structures which extend from the surface of infected protoplasts. *Virology* **195,** 281–285.

Qin, S. W., Ward, B. M., and Lazarowitz, S. G. (1998). The bipartite geminivirus coat protein aids BR1 function in viral movement by affecting the accumulation of viral single-stranded DNA. *J. Virol.* **72,** 9247–9256.

Roberts, A. G., Santa Cruz, S., Roberts, I. M., Prior, D. A. M., and Turgeon, R. (1997). Phloem unloading in sink leaves of *Nicotiana benthamiana*: Comparison of a fluorescent solute with a fluorescent virus. *Plant Cell* **9,** 1381–1396.

Rojas, M. R., Noueiry, A. O., Lucas, W. J., and Gilbertson, R. L. (1998). Bean dwarf mosaic geminivirus movement proteins recognize DNA in a form- and size-specific manner. *Cell* **95,** 105–113.

Rybicki, E. P., Briddon, R. W., Brown, J. K., Fauquet, C. M., Maxwell, D. P., Harrison, B. D., Markham, P. G., Bisaro, D. M., Robinson, D., and Stanley, J. (2000). Family Geminiviridae. *In* "Virus Taxonomy" (van Regenmortel, M. H. V., Fauquet, C. M., and Bishop, D. H. L., Eds.), Classification and nomenclature of viruses. pp. 285–297. Academic Press, San Diego.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Laboratory Harbor Press, Cold Spring Harbor, NY.

Sanderfoot, A. A., Ingham, D. J., and Lazarowitz, S. G. (1996). A viral movement protein as a nuclear shuttle. The geminivirus BR1 movement protein contains domains essential for interaction with BL1 and nuclear localization. *Plant Physiol.* **110,** 23–33.

Sanderfoot, A. A., and Lazarowitz, S. G. (1995). Cooperation in viral movement: The geminivirus BL1 movement protein interacts with BR1 and redirects it from the nucleus to the cell periphery. *Plant Cell* **7,** 1185–1194.

Sanderfoot, A. A., and Lazarowitz, S. G. (1996). Getting it together in plant virus movement: Cooperative interactions between bipartite geminivirus movement proteins. *Trends Cell Biol.* **6,** 353–358.

Scott, A., Wyatt, S., Tsou, P. L., Robertson, D., and Allen, N. S. (1999). Model system for plant cell biology: GFP imaging in living onion epidermal cells. *Biotechniques* **26,** 1125–1132.

Storms, M. M. H., Kormelink, R., Peters, D., Lent, J. W. M. V., and Goldbach, R. W. (1995). The nonstructural NSm protein of tomato spotted wilt virus induces tubular structures in plant and insect cells. *Virology* **214,** 485–493.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.

von Arnim, A., Frischmuth, T., and Stanley, J. (1993). Detection and possible functions of African cassava mosaic virus DNA B gene products. *Virology* **192,** 264–272.

Wang, H. L., Gilbertson, R. L., and Lucas, W. J. (1996). Spatial and temporal distribution of bean dwarf mosaic geminivirus in *Phaseolus vulgaris* and *Nicotiana benthamiana*. *Phytopathology* **86,** 1204–1214.

Ward, B. M., Medville, R., Lazarowitz, S. G., and Turgeon, R. (1997). The geminivirus BL1 movement protein is associated with endoplasmic reticulum-derived tubules in developing phloem cells. *J. Virology* **71,** 3726–3733.

Wege, C., and Jeske, H. (1998). Abutilon mosaic geminivirus proteins expressed and phosphorylated in Escherichia coli. *J. Phytopathol.* **146,** 613–621.

Wege, C., Gotthardt, R.-D., and Jeske, H. (2000). Fulfilling Koch's postulates for Abutilon mosaic virus. *Arch. Virol.* **145,** 2217–2225.

Wege, C., Saunders, K., Stanley, J., and Jeske, H. (2001). Comparative analysis of tissue tropism of bipartite geminiviruses. *J. Phytopathol.* **149,** 359–368.

Wellink, J., van Lent, J. W., Verver, J., Sijen, T., Goldbach, R. W., and van Kammen, A. (1993). The cowpea mosaic virus M RNA-encoded 48-kilodalton protein is responsible for induction of tubular structures in protoplasts. *J. Virol.* **67,** 3660–3664.

Wolf, S., Deom, C. M., Beachy, R. N., and Lucas, W. J. (1989). Movement protein of tobacco mosaic virus modifies plasmodesmata size exclusion limit. *Science* **246,** 377–379.

Wurch, T., Estienne, F., and Pauwels, P. J. (1998). A modified overlap extension PCR method to create chimeric genes in the absence of restriction enzymes. *Biotechniques* **12,** 653–657.