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Tissue and cell tropism of *Indian cassava mosaic virus* (ICMV) and its AV2 (precoat) gene product

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Abstract

In order to establish defined viruses for challenging plants in resistance breeding programmes, *Indian cassava mosaic virus* (ICMV; family *Geminiviridae*) DNA clones were modified to monitor viral spread in plants by replacing the coat protein gene with the green fluorescent protein (GFP) reporter gene. Comparative *in situ* hybridization experiments showed that ICMV was restricted to the phloem in cassava and tobacco. GFP-tagged virus spread similarly, resulting in homogeneous fluorescence within nuclei and cytoplasm of infected cells. To analyze viral intercellular transport in further detail, GFP was fused to AV2, a protein that has been implicated in viral movement. Expressed from replicating viruses or from plasmids, AV2:GFP became associated with the cell periphery in punctate spots, formed cytoplasmic as well as nuclear inclusion bodies, the latter as conspicuous paired globules. Upon particle bombardment of expression plasmids, AV2:GFP was transported into neighboring cells of epidermal tissues showing that the intercellular transport of the AV2 protein is not restricted to the phloem. The results are consistent with a redundant function of ICMV AV2 acting as a movement protein, presumably as an evolutionary relic of a monopartite geminivirus that may still increase virus fitness but is no longer necessary in a bipartite genome. The fusion of ICMV ORF AV2 to the GFP gene is the first example of a reporter construct that follows the whole track of viral DNA from inside the nucleus to the cell periphery and to the next cell.

Keywords: Geminivirus; GFP-tagged viral DNA; Begomovirus; Indian cassava mosaic virus

Introduction

Geminiviruses consisting of circular single-stranded (ss) DNA encapsidated in twin (geminate) particles infect various important crop plants like beet, cassava, maize and tomato with a considerable impact on agriculture and the economy of tropical and subtropical countries. The family *Geminiviridae* comprises four genera, *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus* (Stanley et al., 2005). Some begomoviruses possess one genomic component (monopartite) although most have two components (bipartite; components designated DNA A and DNA B).

The movement of geminiviruses within host plants has been studied extensively (for a review, see Gafni and Epel, 2002; Lazarowitz and Beachy, 1999; Rojas et al., 2005; Waigmann et al., 2004). In the case of bipartite begomoviruses, genes required for systemic spread are located on DNA B (*BV1* syn. *BR1* encoding nuclear shuttle protein, NSP; *BC1* syn. *BL1* encoding movement protein, MP). However, DNA A of some begomoviruses, such as *Abutilon mosaic virus* (AbMV) and *African cassava mosaic virus* (ACMV), may autonomously spread within plants to a certain extent, but without causing symptoms (Evans and Jeske, 1993; Klinkenberg and Stanley, 1990). On the other hand, *Sri Lankan cassava mosaic virus* (SLCMV) DNA A alone is able to fully infect plants although its DNA B modulates symptoms (Saunders et al., 2002).

In contrast to bipartite begomoviruses, monopartite geminiviruses (Boulton et al., 1989; Liu et al., 1998; Rigden et al., 1993) need the coat protein (CP) and the product of a second gene (named *V2*, *V1* or *MP* depending on the virus) preceding

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the *CP* gene for viral spread (Kotlizky et al., 2000; Rojas et al., 2001). The upstream gene product will be referred to as "precoat protein" (PCP) according to Padidam et al. (1996) reflecting its genomic location but not suggesting a function in virus assembly. In the case of bipartite begomoviruses, the presence of PCP is restricted to viruses from the Old World.

The tissue tropism of mono- and bipartite geminiviruses has been investigated extensively. Monopartite and some bipartite begomoviruses are phloem-limited in certain hosts, for example *Tomato yellow leaf curl* virus (TYLCV, Morilla et al., 2004), AbMV and Sida micrantha mosaic-associated viruses (SimMV, Wege et al., 2001), *Bean golden mosaic virus* (BGMV, Carr and Kim, 1983) and *Squash leaf curl virus* (SLCV, Hoefert, 1987). However, some bipartite begomoviruses are able to invade palisade and spongy parenchyma as well as epidermal cells, for example ACMV, *Bean dwarf mosaic virus* (BDMV) and *Tomato golden mosaic virus* (TGMV, Sudarshana et al., 1998; Wege et al., 2001). Tissue tropism seems to be complex in its genetic determination (Morra and Petty, 2000). It has been proposed (Kim and Lee, 1992) that adaptation to the host may be important because Euphorbia mosaic virus (EuMV) is phloem-limited in the natural host (*Euphorbia heterophylla* L.) but less restricted in an experimental host (*Datura stramonium* L.).

Clones of an *Indian cassava mosaic virus* isolate originating from the Indian state of Maharashtra (ICMV-[Mah2]) were recently shown to be infectious in cassava (Rothenstein et al., 2005). The nucleotide sequence of this virus is very similar to that of other cassava-infecting begomoviruses occurring in southern and central India as, for instance, ICMV (Hong et al., 1993) and SLCMV (Rothenstein et al., 2006; Saunders et al., 2002), but more distantly related to that of ACMV (Stanley and Gay, 1983).

In order to alleviate crop losses due to ICMV in India, it is desirable to establish reliable tools, including infectious clones for resistance screening in breeding programmes. To this aim, we investigated the tissue tropism of cassava-infecting begomoviruses in natural and experimental hosts by *in situ* hybridization and constructed recombinant ICMV clones expressing green fluorescent protein (GFP). The results show that ICMV is phloem-limited in cassava and *Nicotiana* spp. and suggest a role for AV2 in viral movement.

Results

Distribution of wild-type viral DNA within source plants

The tissue tropism of begomoviruses in naturally infected cassava and experimentally infected tobacco plants was compared by use of *in situ* hybridization at the light-microscopic level. Specimens of leaves and petioles were hybridized with biotin-labelled ICMV-[Mah2] DNA A-specific probes, resulting in precipitation of a blue stain (Figs. 1 and 2). Signals were more abundant and pronounced in tobacco than in cassava and absent in healthy control plants (not shown). With only one exception (one case in 200 sections analyzed which all showed viral signals; Fig. 1b), where signals appeared in spongy parenchyma in several adjacent cells, ICMV DNA was generally found to be phloem-limited. Although plants suffered from severe symptoms, few infected cells were detected and these were generally surrounded by apparently virus-free cells.

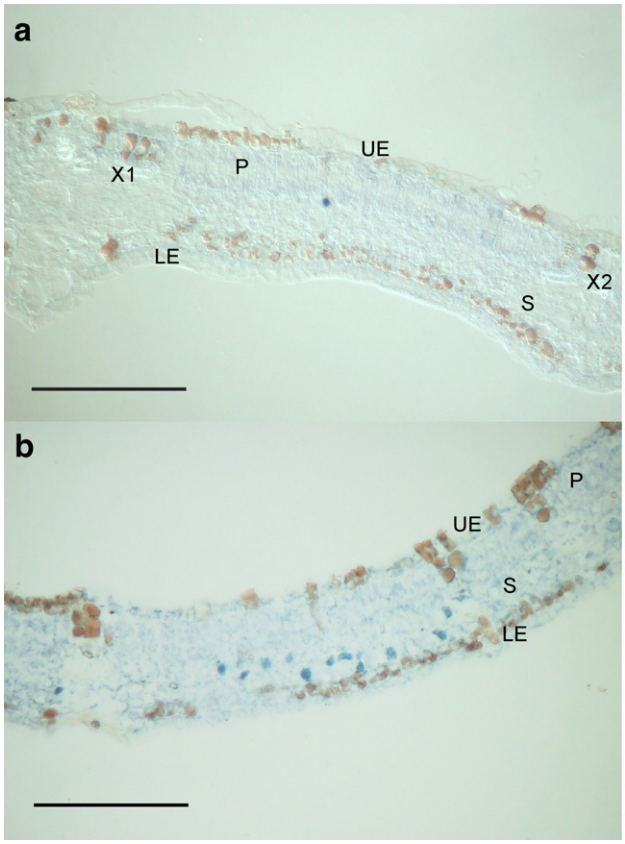

Fig. 1. *In situ* hybridization of viral DNA in naturally infected cassava using biotin-labelled ICMV-[Mah-2] DNA A probes and a blue staining TMB substrate. Cross-section through leaves showing the common case in panel a and a rare case in panel b. Typically, single isolated signals (blue spot) were associated with the veins, the section between X1 (xylem element 1) and X2 (xylem element 2) of which was cut away in panel a, whereas palisade parenchyma (P), spongy parenchyma (S), upper epidermis (UE) and lower epidermis (LE) were free of specific signals. In contrast, signals cluster in adjacent cells of the spongy parenchyma in panel b. Scale bar represents 100 μm.

Similarly, virus was phloem-limited in tobacco (Fig. 2). Although more abundant than in cassava, no arrays of adjacent infected cells indicative of lateral virus cell to cell movement were observed.

Distribution of recombinant ICMV-[Mah2] DNA A

Nicotiana benthamiana plants were inoculated with three recombinant ICMV-[Mah2] DNA A constructs, ΔCPΔAV2-GFP, ΔCP-GFP and ΔCP-AV2:GFP, each together with wild-type (wt) ICMV-[Mah2] DNA B (Fig. 3). Symptoms developed in infections with all recombinant constructs within 19 to 22 days post-inoculation (dpi), which was 4–7 days delayed compared to wt ICMV (three independent experiments). Plants infected with recombinant viruses displayed less pronounced symptoms, exhibited crinkling and faint yellowing of the larger leaves. The amounts of viral DNA were significantly reduced

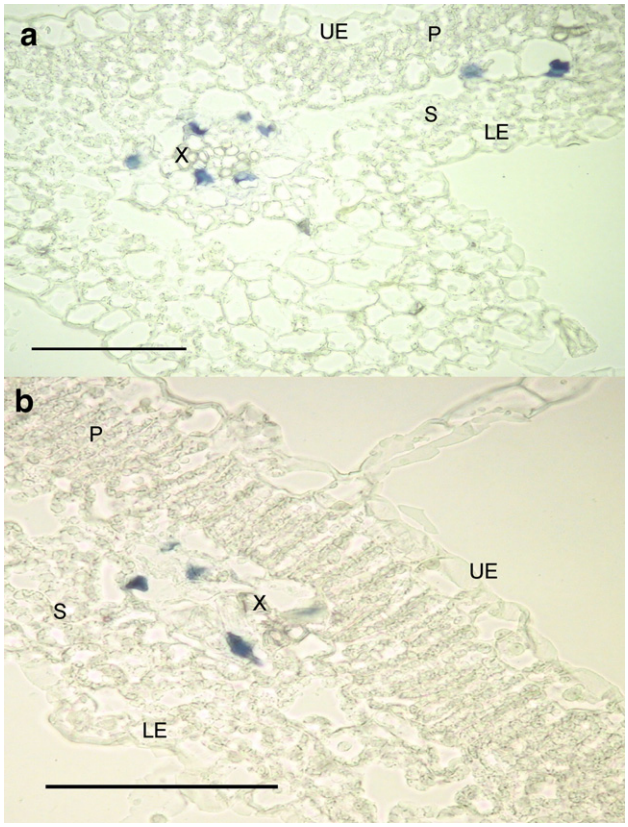

Fig. 2. *In situ* hybridization of viral DNA as in Fig. 1 but for experimentally ICMV-infected tobacco. Two representative cross-sections of infected leaves show the association of specific signals with veins by their vicinity to the xylem (X) and their absence in other tissues. Abbreviations as in Fig. 1. Scale bar represents 100 μm.

for all recombinant viruses (Fig. 3c), with approximately equal portions of ss and dsDNA for constructs with an intact ORF AV2, compared to a higher proportion of ssDNA produced by wt virus and a lower proportion of ssDNA for the construct with a disrupted ORF AV2. The differences in sizes of the chimeric ICMV DNA A constructs were retained during the infection process. It is noticeable that the amount of ssDNA was also decreased for DNA B (Fig. 3c, lanes 2–4). *GFP* was stably maintained during the course of experiments showing that the constructs were within the size range tolerated without the need for size reversion as has been observed for significantly smaller and larger components (Bisaro, 1994; Etessami et al., 1989; Gilbertson et al., 2003).

Leaves and stems of systemically infected *N. benthamiana* plants were investigated by epifluorescence microscopy. ΔCPΔAV2-GFP-infected plants showed fluorescence of free GFP exclusively associated with the vascular system of leaves (Fig. 4a). As expected for an unfused reporter protein (Zhang et al., 2001), GFP was homogeneously distributed throughout the cytoplasm and accumulated within nuclei of those cells.

When GFP was fused to AV2 (ΔCP-AV2:GFP), fluorescence was similarly associated with the vascular system (Fig. 4d, e) and predominated in sink portions of leaves where AV2:GFP accumulated in class I to III veins (Fig. 4e). At higher magnification (Fig. 4b, c), the fluorescent signals were detected as prominent cytoplasmic inclusions (CI) which were responsible for most of the fluorescence seen in the overview of Fig. 4d, e. In addition, discrete punctate spots were located at the cell periphery and bright globules within the nucleus (Fig. 4d, white arrowhead, as identified below in closer detail).

ICMV-[Mah2] DNA A(ΔCP-GFP) did not show any GFP fluorescence, although it replicated efficiently and stably (Fig. 3c). Presumably, the AUG codon of GFP downstream of the CP AUG is not recognized properly according to Kozak's (1991) rule or reinitiation simply does not occur immediately downstream of the AV2 stop codon.

Distribution of AV2:GFP upon transient expression

As a control, unfused GFP (Fig. 4f) was expressed from replicating ICMV A (ΔCPΔAV2-GFP) and showed its typical uniform distribution with an accumulation in the nucleus as expected (Zhang et al., 2001). Under these conditions, little GFP leaked out to the neighboring cells, a transport which was expected from earlier experiments using GFP in sink leaves but not for enlarged GFP fusions (Zhang et al., 2002). In addition to serve as controls, this experiment shows that the promoter in front of AV2 and CP is active in epidermis cells and not restricted to phloem cells.

The spread of transiently expressed AV2 fused to GFP was followed after particle bombardment of non-replicating plasmid constructs. *N. benthamiana* leaves of various sizes were bombarded with pGFP:AV2. In small leaves (1 to 3 cm in length, sink state) and leaves undergoing the sink-to-source transition (3 to 4 cm in length from leaf base to leaf tip), GFP fluorescence appeared 8 to 10 h post-bombardment (hpi) and was, at that time, restricted to single epidermal cells. The majority of cells (~90%; 990 out of 1100 GFP-expressing cells) usually showed one bright inclusion body of GFP:AV2 (Fig. 4g, h; open arrowhead) as well as several discrete punctate spots and stretches of fluorescence along the cell periphery (Fig. 4g, h). Although the inclusion bodies frequently resembled nuclei, counterstaining with DAPI clearly identified them to be located outside the nucleus, presumably within the cytoplasm (Fig. 4g, h, open arrowhead). Within the nuclei, on the other hand, one or two GFP globules were frequently present, which resemble nucleoli in size and pairwise appearance (Fig. 4g, h; inserts) but must have been of different origin because they were intensely labelled by DAPI. Round-shaped DAPI-negative areas, typical of nucleoli, were found at different sites within the same nuclei (Fig. 4h, insert, black arrowhead). In approximately 20% of the cells (~200 out of 1100 GFP-expressing cells), all AV2:GFP signals formed punctate spots at the cell periphery (data not shown).

Nuclear inclusions (NI) appeared only early after bombardment (18–25 hpb) and disappeared later on. To quantify their occurrence, two independent bombardments each were done on *N. benthamiana* and *Nicotiana tabacum* Samsun nn sink leaves, revealing 44 fluorescing cells without compared to 22 with nuclear localization for the former and 118 versus 32 for the latter plant species. Within the class of NI-containing cells, 10 exhibited one, 12 two globules per nucleus for *N. benthamiana*

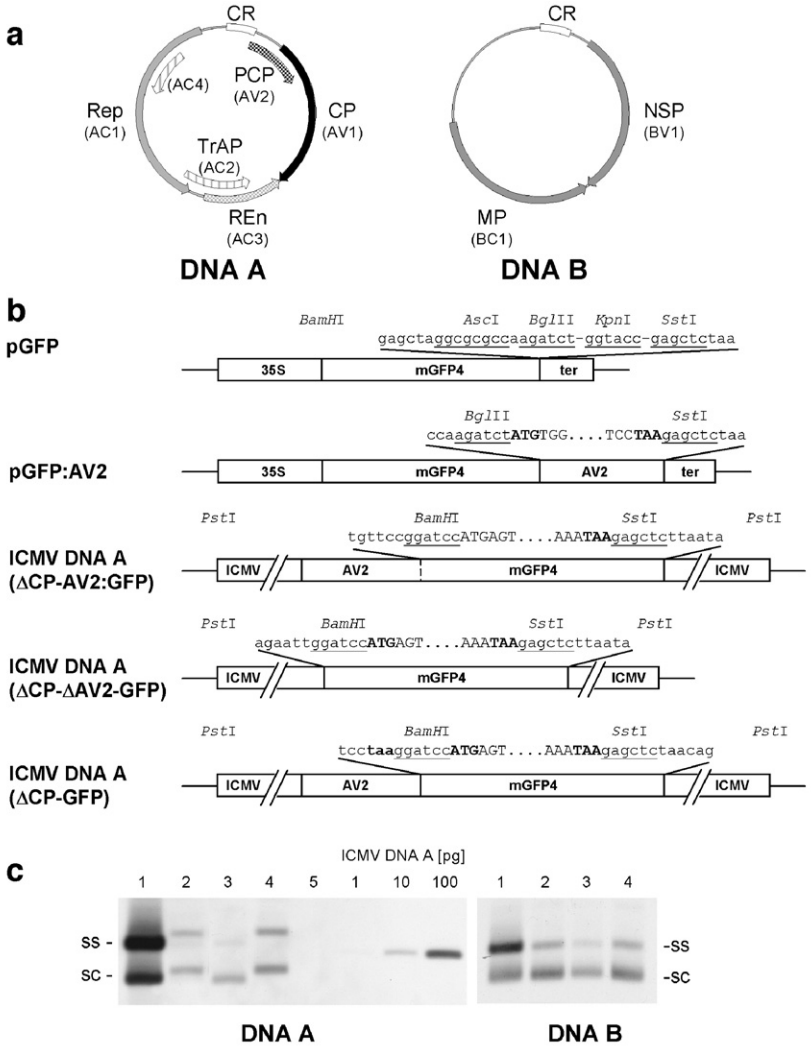

Fig. 3. (a) Genome organization of *Indian cassava mosaic virus* (ICMV-[Mah2]) DNA A and DNA B with open reading frames, assigned gene functions (Rep: replication-associated protein; TrAP: transcriptional activator protein; REn: replication enhancer; CP: coat protein; PCP: precoat protein; MP: movement protein; NSP: nuclear shuttle protein) and common region (CR) indicated. (b) Recombinant green fluorescent protein (GFP) reporter constructs of ICMV-[Mah2] used in this study. Note that ORF AV2 is truncated in DNA A(ΔCPΔAV2-GFP) whereas DNA A(ΔCP-GFP) and (ΔCP-AV2:GFP) retain the entire ORF AV2 without or with fusion to the GFP gene, respectively. 35S indicates *Cauliflower mosaic virus* (CaMV) 35S promoter, ter indicates nopaline synthase terminator. Recognition sequences of restriction sites used for cloning are underlined. Start and stop codon are printed in bold face, upper cases indicate coding start and stop region of GFP. (c) Southern blot analysis of wt ICMV-[Mah2] and recombinant GFP constructs in systemically infected *N. benthamiana* plants. Plants biolistically inoculated with wt (lane 1), (ΔCP-AV2:GFP) (lane 2), (ΔCPΔAV2-GFP) (lane 3), (ΔCP-GFP) (lane 4) or without DNA (lane 5) were harvested 20 dpi and 3 μg of extracted nucleic acids were separated on an 1% agarose gel. Cloned linear ICMV-[Mah2] DNA A (1, 10 and 100 pg per lane) served as hybridization standard. Parallel blots were either probed for DNA A (left) or DNA B (right). Positions of single-stranded (ss) and supercoiled (sc) DNA forms are indicated.

and 26 one, 2 two, 3 three and 1 four globules for *N. tabacum*. These numbers may be biased to smaller values, because some globules were hiding each other.

The nuclear localization of GFP:AV2 was further scrutinized using confocal laser scanning microscopy (Fig. 5). After screening several nuclei through optical sections, no doubt remained that the NI globules were within the nuclei but outside of nucleoli. The same analysis confirmed the peripheral sites of punctate spots and the cytoplasmic location of the large inclusions (CI).

During the progress of transient expression, fluorescence became detectable in adjacent cells, where it appeared first at 18 hpi and later in up to 60% of the primary expressing cells at 48 hpi. GFP:AV2 moved into up to four additional cells. With our experimental conditions (Zhang et al., 2001, 2002), a translocation of GFP was generally rare in case of the unfused protein and absent if GFP was fused to proteins larger than 10 kDa (compared to 13.6 kDa for PCP). Such a high frequency of fluorescence in neighboring cells, as observed here for AV2:GFP, did not occur either for constructs expressing either free GFP (this study), or for other GFP fusion constructs in previous studies (Unseld et al., 2001; Zhang et al., 2001, 2002). These results substantiate the notion that AV2 is able to promote cell–cell transport of the GFP reporter.

The probability of co-bombarded adjacent cells occurring by chance using our experimental procedure has been quantified in

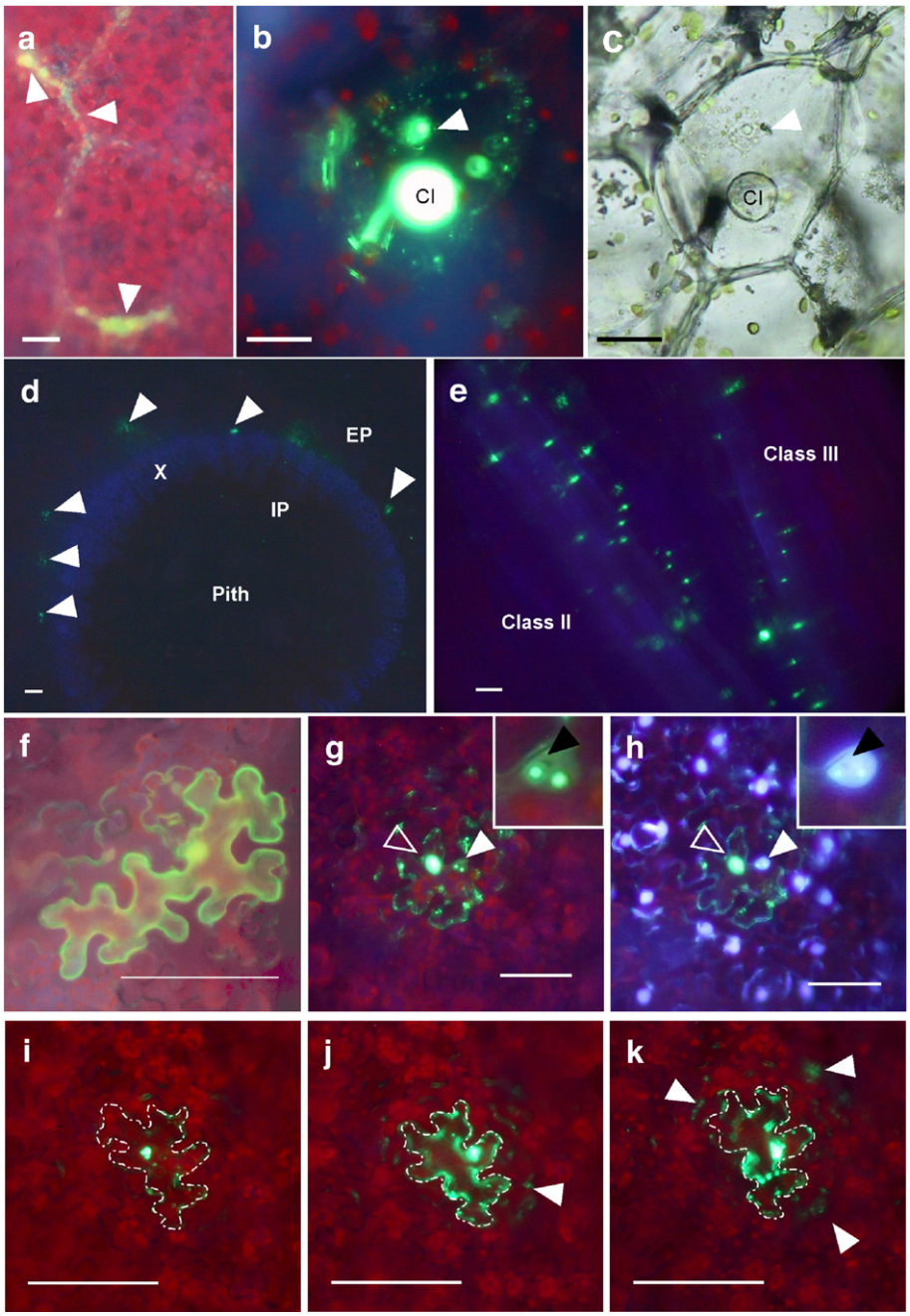

Fig. 4. GFP expression in *N. benthamiana* either in systemically infected leaves (a–e), in leaves bombarded with the infectious DNA A ΔCPΔAV2-GFP (f) expressing free GFP under the control of the coat protein promoter or with plasmids pGFP:AV2 (g-k) expressing GFP:AV2 fusion protein under the control of the 35S promoter. Fluorescence of unfused GFP from ICMV-[Mah2] DNA A (ΔCPΔAV2-GFP) showed evenly distributed signals along the vasculature of the leaf (a). In contrast, AV2:GFP fusion protein expressed from ICMV ΔCP-AV2:GFP was localized to discrete fluorescent foci in stem sections (d) and lower surface views of leaf veins (e) close to xylem (X), which exhibited a blue autofluorescence. EP: external phloem; IP: internal phloem. At higher magnification (b, c), the same phloem parenchyma was visualized by fluorescence (b) or by bright field microscopy (c) to show the cellular localization of the AV2:GFP fluorescent signals. A prominent cytoplasmic inclusion (CI) was accompanied by a less intense fluorescence within the nucleus (white arrowheads), and tiny punctate spots at the cell periphery. Note the brighter globular fluorescent spot within the nucleus. A similar variability in subcellular localization was observed when unfused GFP (f) or fused GFP:AV2 (g–k) were expressed in bombarded leaves. Epidermal cells of sink leaves exhibited free GFP in cytoplasm and nucleus (f) or AV2-fused GFP at the cell periphery, in cytoplasmic inclusions and within the nucleus (g–k). Large GFP:AV2 cytoplasmic inclusion bodies were formed (g; 18 hpi, open arrowheads) outside the nucleus, as shown by DAPI-counterstaining of the same cell (h). Moreover, small nuclear green-fluorescent globules were present within the nuclei that were also stained with DAPI (g, h; filled white arrowheads; inserts show the nuclei at higher magnification; black arrowhead points at non-fluorescent nucleoli). Note that GFP:AV2 was already present in cells neighboring adjacent to the bombarded cell. To follow the development of the fluorescence over time, the same epidermal cell was photographed at 10 (i), 24 (j) and 48 (k) hpi, the initially bombarded cell being marked by white lines. Detected from 24 hpi, GFP:AV2 has spread to neighboring cells (j, k; white arrowheads). Scale bars represent 2 μm in panels b and c; and 5 μm in all other images.

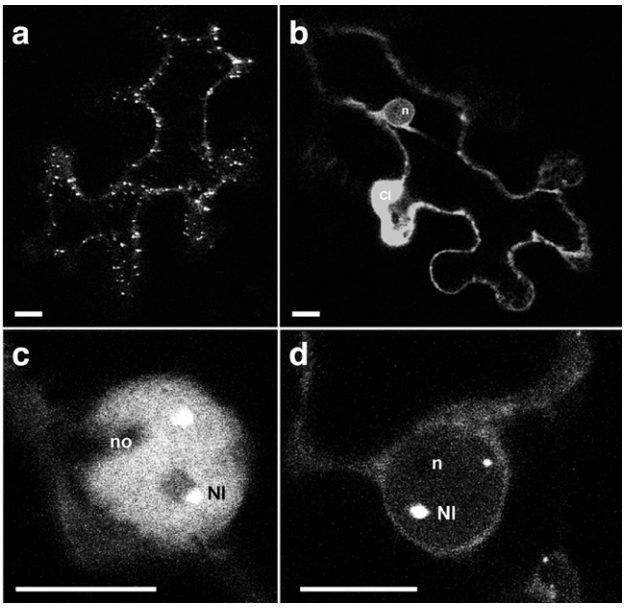

Fig. 5. Confocal laser scanning micrographs of GFP:AV2 expressing epidermis cells following particle bombardment with pGFP:AV2. Optical sections using the GFP channel confirm the cellular localization of peripheral punctate spots (a), cytoplasmic inclusions (CI) and nuclear inclusions (NI; c, d) leaving the nucleoli (no) as dark areas. Scale bars represent 10 μm.

great detail (Zhang et al., 2001) and was found to be extremely low. Nevertheless, to exclude this possibility, GFP:AV2 distribution was monitored in the same cell over a period of 48 h. At the first time point (10 hpb), fluorescence accumulated in the cytoplasm and in punctate spots at the cell periphery (Fig. 4i). The intensity of fluorescence increased by 24 hpb in the initially expressing cell, and concomitantly first weak signals developed in neighboring cells (Fig. 4j). Fluorescence had further increased in the neighboring cells by 48 hpb (Fig. 4k).

Discussion

Although ACMV is not restricted to the phloem in its experimental host *N. benthamiana* (Wege et al., 2001), several lines of evidence reported here allow us to conclude that ICMV is confined to phloem in the natural host cassava as well as in the experimental host *N. tabacum*. Tissue blot analysis showed a similar phloem restricted localization of ICMV in *N. benthamiana* (D. Haible, unpublished data). The results are consistent with earlier electron microscopy and immunolabelling of ICMV in *N. benthamiana* plants (Roberts, 1989). Why some geminiviruses are phloem-limited and others are not does not follow any regularity concerning host plant, genomic structure or transmission mode. It is still unclear which factors are relevant, and possibly multiple explanations are valid (see discussion in Morilla et al. (2004, and references therein). At least three mechanisms may underlie the phenomenon: (a) viral replication and transcription may vary in cells outside the phloem, (b) efficiency of movement proteins may alter in different tissues and (c) differential defence strategies of the host may act in different tissues. Geminiviruses do not encode a DNA polymerase but rely on the cellular replication machinery. They stimulate plant cells to enter into S-phase (Nagar et al., 2002) and induce expression of proliferating cell nuclear antigen (PCNA), the processivity factor of DNA polymerase δ (Nagar et al., 1995). Impaired interaction of TGMV AC1 with a plant retinoblastoma-(Rb)-like protein (pRBR), a cell cycle regulator, caused phloem restriction of TGMV (Kong et al., 2000), clearly demonstrating that this gene product significantly affects tissue tropism. In addition, viral promoter elements may function in a tissue-specific manner, for example causing activation and derepression of virion-sense genes as demonstrated for TGMV (Sunter and Bisaro, 1997). However, we demonstrated that recombinant ICMV-[Mah2] DNA A expressed high levels of GFP in epidermal cells under the control of the geminiviral CP promoter (Fig. 4f). Movement proteins may be less able to facilitate transport of viral DNA through the specialized plasmodesmata of non-phloem cells. For example, TGMV mutants with point mutations in BC1 showed reduced invasion into these cells although they were not completely phloem-limited and infected palisade and spongy parenchyma cells (Saunders et al., 2001). Finally, restriction of viruses to the vascular system may be caused by host defence mechanisms such as transcriptional and posttranscriptional gene silencing. The *AC2* and *AC4* gene products have been implicated in silencing suppression which may be functionally tissue dependent (van Wezel et al., 2002, 2003; Vanitharani et al., 2004; Voinnet et al., 1999).

Accumulating evidence suggests that geminiviruses use two different transport strategies. Monopartite geminiviruses exploit the interaction of precoat protein (PCP) with coat protein (CP), whereas bipartite begomoviruses acquired the genes on DNA B, *NSP* and *MP*, to provide cell–cell transport. Within the genus *Begomovirus*, both strategies have been adopted as some members are monopartite, others bipartite. Moreover, transitional states have been observed where the DNA A component alone is sufficient to induce infection but the DNA B component modulates pathogenesis (Saunders et al., 2002). These cases suggest a continuous evolution of geminiviruses. For ICMV-[Mah2] (this study), PCP is not absolutely necessary for full infection but its cellular localization is, nevertheless, consistent with a function in movement, similar to the PCPs of monopartite begomo- and mastreviruses (Kotlizky et al., 2000; Padidam et al., 1996; Rojas et al., 2001; Wartig et al., 1997). The punctate appearance of ICMV PCP at the cell periphery is similar to that of many plant virus movement proteins which accumulate at the plasmodesmata (Waigmann et al., 2004).

On the other hand, ICMV-[Mah2] possesses a DNA B component that is necessary for systemic infection (Rothenstein et al., 2005). We propose that PCP may be an evolutionary relic of monopartite parents and provides a redundant movement function that may still increase the fitness of the virus in nature but is no longer necessary for its movement under experimental conditions. Although phloem-restricted if expressed from a replicating virus, PCP is transported from cell to cell in epidermal tissues. Similar results were obtained with AbMV proteins NSP and MP. Whereas AbMV is strictly phloem-limited, its BC1 and BV1 proteins were co-transported if co-expressed in epidermal cells of sink leaves (Zhang et al., 2001).

Similar studies on ICMV BC1 and BV1 are under progress in order to ascertain whether a functionally impaired movement protein may be responsible for its phloem limitation. The intracellular distribution of ICMV PCP differs from that of AbMV DNA B-encoded MP in one aspect. Whereas AbMV MP was found to be located around, but never within nuclei (Zhang et al., 2001, 2002), ICMV ORF AV2:GFP expressed globular inclusions within the nucleoplasm which may be mistaken for nucleoli as discussed above. Similar globules, however, have been detected for AbMV NSP, the second DNA B-encoded gene involved in movement (Zhang et al., 2001). In addition, globular nuclear inclusions in the vicinity of nucleoli are also formed by accumulating AbMV virions as shown by immunolabelling on ultrathin plant tissue sections (Abouzid et al., 1988). The pairwise appearance of nuclear ICMV AV2:GFP globules, their rounded shape and DNA content resembles similar nuclear but extra-nucleolar structures, the so-called "Cajal bodies" (Gall, 2000). Establishing foci of viral proteins within the nuclei may be a more general strategy involved in multiplication and transport for different purposes. In this context, it is interesting to note that even an RNA virus like *Cucumber mosaic virus* (CMV), which replicates within the cytoplasm, expresses a movement protein that translocates to the nucleus and forms nucleoli-like aggregates (Mackenzie and Tremaine, 1988). Whereas a function for CMV MP in counteracting host defence has been proposed, it is tempting to speculate that the occurrence of ICMV PCP within the nucleus may reflect an ancient PCP-mediated transport route of geminiviruses, which was subsequently accomplished by NSP and MP in bipartite geminiviruses.

Whether the large, brightly fluorescing cytoplasmic inclusion bodies represent functional states of PCP appears rather questionable. Similar protein aggregates were frequently found upon over-expression or misfolding of proteins and may indicate waste disposal rather than functional structures (Kopito, 2000).

By using the GFP-tagged ICMV constructs obtained and characterized in this study, it will now be possible to challenge large numbers of cassava cultivars to screen for resistance at the cellular and tissue level using simple inoculation techniques (Rothenstein et al., 2005) and fluorescence microscopy.

Materials and methods

Plants and viruses

Stem cuttings from symptomatic cassava plants (*Manihot esculenta* Crantz.) were collected in the southern Indian states of Tamil Nadu and Kerala in 2002 (Rothenstein et al., 2006). Regenerated cuttings were grown in an insect-free glasshouse at 24 °C with supplementary illumination. *Begomovirus* infection was verified by Southern blot analysis using ICMV DNA A-specific probes. *N. tabacum* L. cv. Samsun nn plants at the two-to-three leaf stage were inoculated with ICMV-[Mah2] clones (Rothenstein et al., 2005, accession numbers: AY730035/AY730036) by particle bombardment as described (Jovel et al., 2004). Infection had established 14 to 17 days post-inoculation (dpi) and was confirmed by Southern blot analysis. *N. benthamiana* plants were sap-inoculated using systemically infected cassava leaves ground in a mortar and extracted with 1 ml of water as inoculum. 10-µl aliquots were rub-inoculated with the aid of Carborundum powder as abrasive. Infection was established at 15–18 dpi and confirmed by Southern blot analysis.

In situ hybridization and detection

Leaf and petiole explants from begomovirus-infected cassava and tobacco plants, approximately 3 to 4 mm in diameter, were fixed, paraffin-embedded, sectioned, hybridized using biotin-labelled probes and virus was detected using tetramethylbenzidine (TMB), resulting in a blue precipitate (Morilla et al., 2004). Probes were generated by polymerase chain reaction (PCR) amplification of a virus-specific DNA fragment covering the *CP* gene, using cloned ICMV-[Mah2] DNA A template and degenerate primers #1 and #2 (Table 1).

Table 1
Primers used for cloning and verification of ICMV/GFP constructs

#	Primer	Sequence [a]	Location [b]
1	ICMV CP for	5′-ATGTCGAAGCGACCAGSAGATATWAT-3′	ICMV A (v) nt 295–320
2	ICMV CP rev	5′-TTAATTKSTCACTGMATCATAGAARTA-3′	ICMV A (c) nt 1065–1039
3	AV2-ICMV rev	5′-TAAT*GGATCC*GGAACATCTGGGCTTCTGTA-3′	ICMV A (c) nt 489–469
4	AV2-ICMV for	5′-CTTCTATGATTCAGT*GAGCTC*TAATAAATA-3′	ICMV A (v) nt 1041–1071
5	GFP *Bam*HI	5′-ATA*GGATCC*ATGAGTAAAGGAGAAGAAC-3′	*mgfp4* nt 1–19
6	GFP *Sst*I	5′-CGT*GAGCTC*TTATTTGTATAGTTCATCC-3′	*mgfp4* nt 719–737
7	ΔAV1/AV2 rev	5′-AGC*GGATCC*AATTCTTCGCCCTAATAACAG-3′	ICMV A (c) nt 300–274
8	ΔAV1stop rev	5′-CCTTCA*GGATCC*TTAGGAACATCTGGGCTT-3′	ICMV A (c) nt 503–474
9	AV2-for *Bgl*II	5′-GTTGCC*AGATCT*ATGTGGGACCCTTTACTA-3′	ICMV A (v) nt 123–157
10	AV2-rev *Sst*I	5′-CCTTCA*GAGCTC*TTAGGAACATCTGGGCTT-3′	ICMV A (c) nt 503–474
11	GFP-rev [c]	5′-GTATGTTGCAGCACCTTCACCC-3′	*mgfp4* nt 116–137
12	GFP-for [c]	5′-ATTACCTGTCCACACAATCTG-3′	*mgfp4* nt 596–616

[a] Restriction sites introduced by PCR are italicized.
[b] *gfp*: numbering starts at start codon; ICMV A: numbering according to Rothenstein et al., 2005; (v) in viral or (c) in complementary orientation.
[c] Sequencing primer.

Construction of recombinant ICMV-[Mah2] DNA-A constructs

Recombinant DNA techniques were performed according to Sambrook and Russell (2001). Restriction endonucleases and DNA-modifying enzymes were used as recommended by the manufacturers. Cloning of monomeric ICMV-[Mah2] DNA A into pBluescript has been described (Rothenstein et al., 2005). To construct recombinant DNA A, restriction sites *Bam*HI and *Sst*I within the multiple cloning site of the plasmid were removed by digestion with these enzymes and mung bean nuclease, followed by religation. In all cases, the *mgfp4* gene (Haseloff et al., 1997) was used to obtain soluble GFP after expression. To construct ΔCP-AV2:GFP, nucleotides 489 to 1062 of ICMV-[Mah2] DNA A, including the *AV2* stop codon and the *CP*, were removed and *Bam*HI and *Sst*I sites were added using PCR with primers #3 and #4 (Table 1), which amplified the truncated ICMV and plasmid DNA. The PCR product was digested with *Bam*HI and *Sst*I and gel purified resulting in the fragment pICMVΔCP. The *GFP* gene was amplified from *mgfp4* (Haseloff et al., 1997) using primers #5 and #6, adding *Bam*HI and *Sst*I sites, respectively (Table 1). Fragments were digested with *Bam*HI and *Sst*I, gel purified and ligated to pICMVΔCP to give ΔCP-AV2:GFP (Fig. 3), which replicated slightly larger DNA (2902 nts) than wt DNA A (2733 nts) *in planta*.

The same strategy was used to produce ΔCP-ΔAV2-GFP (Fig. 3), with nucleotides 395–1062 deleted using primers #7 and #4 (Table 1), and ΔCP-GFP (Fig. 3), with nucleotides 491–1062 deleted, conserving the stop codon between *AV2* and *GFP* by use of primers #8 and #4. Both PCR products were digested with *Bam*HI and *Sst*I, and *GFP* was inserted. *In planta* replication products derived from ICMV-[Mah2] DNA A (ΔCPΔAV2-GFP) (2808 nts) and ICMV-[Mah2] DNA A (ΔCP-GFP) (2905 nts) were slightly larger in size than wt DNA A (2733 nts).

In order to study AV2 function in the absence of other viral genes, pGFP:AV2 (Fig. 3) was constructed. *AV2* was PCR-amplified using primers #9 and #10 (Table 1) adding a *Bgl*II site at the 5′-end and an *Sst*I site at the 3′-end. This fragment was digested with *Bgl*II and *Sst*I and introduced into the *Bgl*II and *Sst*I sites of *gfp*, resulting in pGFP:AV2 (Fig. 3). The sequence integrity of all the four constructs was confirmed by automated sequencing as described (Jovel et al., 2004), using fluorescently labelled (IRD 800) primers #11 and #12.

Inoculation of plants

To establish systemic infections, ICMV-[Mah2] DNA A (ΔCP-AV2:GFP) was released from the plasmid by *Pst*I digestion and mixed with equal amounts of ICMV-[Mah2] DNA B released from its plasmid by *Kpn*I. *N. benthamiana* plants at the two-leaf stage were inoculated with DNA by particle bombardment (450 psi) as described (Zhang et al., 2001). For transient expression, undigested pGFP:AV2 DNA or, as a control, p35S:GFP were similarly inoculated onto leaves of various sizes (Zhang et al., 2001). GFP was monitored using an epifluorescence microscope equipped with filter cube Zeiss 02 (BP 450–490; FT 510; LP 520; Zeiss Axiophot). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Zhang et al., 2001).

Confocal laser scanning microscopy

Leaves were prepared from GFP:AV2 expressing plants and analyzed using a confocal laser scanning microscope (TCS SP2, Leica Microsystems, Germany).

The leaves were mounted on glass slides in double distilled water, covered with a coverslip and imaged with a 63×/1,2 HCX PL Apo W Corr objective lens. GFP was excited with the 488-nm line of the argon laser (AOTF between 20% and 25%) and fluorescence was detected at 500 nm to 600 nm (Fig. 5a, c, d) or 500 to 550 nm (Fig. 5b). Confocal sections were acquired every 162 nm (Fig. 5a, c, d) or 285 nm (Fig. 5b). Images were processed with Image J (NIH, USA) (LUT level adjusted and slightly gamma-corrected for better contrast) and prepared for presentation with Adobe Photoshop (Adobe, USA).

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References

Abouzid, A.M., Barth, A., 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.

Bisaro, D.M., 1994. Recombination in geminiviruses: mechanisms for maintaining genome size and generating genomic diversity. In: Paszkowski, J. (Ed.), Homologous Recombination and Gene Silencing in Plants. Kluwer Academic Publishers, Dordrecht, pp. 39–60.

Boulton, M.I., Steinkellner, H., Donson, J., Markham, P.G., King, D.I., Davies, J.W., 1989. Mutational analysis of the virion-sense genes of maize streak virus. J. Gen. Virol. 70, 2309–2323.

Carr, R.J., Kim, K.S., 1983. Evidence that bean golden mosaic virus invades non-phloem tissue in double infections with tobacco mosaic virus. J. Gen. Virol. 64, 2489–2492.

Etessami, P., Watts, J., Stanley, J., 1989. Size reversion of African cassava mosaic virus coat protein gene deletion mutants during infection of *Nicotiana benthamiana*. J. Gen. Virol. 70, 277–289.

Evans, D., 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.

Gafni, Y., Epel, B.L., 2002. The role of host and viral proteins in intra- and inter-cellular trafficking of geminiviruses. Mol. Plant Pathol. 60, 231–241.

Gall, J.G., 2000. Cajal bodies: the first 100 years. Annu. Rev. Cell Dev. Biol. 16, 273–300.

Gilbertson, R.L., Sudarshana, M., Jiang, H., Rojas, M.R., Lucas, W.J., 2003. Limitations on geminivirus genome size imposed by plasmodesmata and virus-encoded movement protein: insights into DNA trafficking. Plant Cell 15, 2578–2591.

Haseloff, J., Siemering, K.R., Prasher, D.C., 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. U. S. A. 94, 2122–2127.

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

Hong, Y.G., Robinson, D.J., Harrison, B.D., 1993. Nucleotide sequence evidence for the occurrence of three distinct whitefly-transmitted geminiviruses in cassava. J. Gen. Virol. 74, 2437–2443.

Jovel, J., Reski, G., Rothenstein, D., Ringel, M., Frischmuth, T., Jeske, H., 2004. Sida micrantha mosaic is associated with a complex infection of begomoviruses different from Abutilon mosaic virus. Arch. Virol. 149, 829–841.

Kim, K.S., Lee, K.W., 1992. Geminivirus-induced macrotubules and their suggested role in cell-to-cell movement. Phytopathology 82, 664–669.

Klinkenberg, F.A., Stanley, J., 1990. Encapsidation and spread of African cassava mosaic virus DNA A in the absence of DNA B when agroinoculated to *Nicotiana benthamiana*. J. Gen. Virol. 71, 1409–1412.

Kong, L.J., Orozco, B.M., Roe, J.L., Nagar, S., Ou, S., Feiler, H.S., Durfee, T., Miller, A.B., Gruissem, W., Robertson, D., Hanley-Bowdoin, L., 2000. A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. EMBO J. 19, 3485–3495.

Kopito, R.R., 2000. Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol. 10, 524–530.

Kotlizky, G., Boulton, M.I., Pitaksutheepong, C., Davies, J.W., Epel, B.L., 2000. Intracellular and intercellular movement of maize streak geminivirus V1 and V2 proteins transiently expressed as green fluorescent protein fusions. Virology 274, 32–38.

Kozak, M., 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. 266, 19867–19870.

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

Liu, L., Davies, J.W., Stanley, J., 1998. Mutational analysis of bean yellow dwarf virus, a geminivirus of the genus *Mastrevirus* that is adapted to dicotyledonous plants. J. Gen. Virol. 79, 2265–2274.

Mackenzie, D.J., Tremaine, J.H., 1988. Ultrastructural location of non-structural protein 3A of cucumber mosaic virus infected tissue using monoclonal antibodies to a cloned chimeric fusion protein. J. Gen. Virol. 69, 2387–2395.

Morilla, G., Krenz, B., Jeske, H., Bejarano, E.R., Wege, C., 2004. Tête à tête of Tomato yellow leaf curl virus (TYLCV) and Tomato yellow leaf curl Sardinia virus (TYLCSV) in single nuclei. J. Virol. 78, 10715–10723.

Morra, M.R., Petty, I.T., 2000. Tissue specificity of geminivirus infection is genetically determined. Plant Cell 12, 2259–2270.

Nagar, S., Pedersen, T.J., Carrick, K.M., Hanley-Bowdoin, L., Robertson, D., 1995. A geminivirus induces expression of a host DNA synthesis protein in terminally differentiated plant cells. Plant Cell 7, 705–719.

Nagar, S., Hanley-Bowdoin, L., Robertson, D., 2002. Host DNA replication is induced by geminivirus infection of differentiated plant cells. Plant Cell 14, 2995–3007.

Padidam, M., Beachy, R.N., Fauquet, C.M., 1996. The role of AV2 ("precoat") and coat protein in viral replication and movement in tomato leaf curl geminivirus. Virology 224, 390–404.

Rigden, J.E., Dry, I.B., Mullineaux, P.M., Rezaian, M.A., 1993. Mutagenesis of the virion-sense open reading frames of tomato leaf curl geminivirus. Virology 193, 1001–1005.

Roberts, I.M., 1989. Indian cassava mosaic virus: ultrastructure of infected cells. J. Gen. Virol. 70, 2729–2739.

Rojas, M.R., Jiang, H., Salati, R., Xoconostle-Cazares, B., Sudarshana, M.R., Lucas, W.J., Gilbertson, R.L., 2001. Functional analysis of proteins involved in movement of the monopartite begomovirus. Tomato yellow leaf curl virus. Virology 291, 110–125.

Rojas, M.R., Hagen, C., Lucas, W.J., Gilbertson, R.L., 2005. Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. Annu. Rev. Phytopathol. 43, 361–394.

Rothenstein, D., Briddon, R.W., Haible, D., Stanley, J., Frischmuth, T., Jeske, H., 2005. Biolistic infection of cassava using cloned components of Indian cassava mosaic virus. Arch. Virol. 150, 1669–1675.

Rothenstein, D., Haible, D., Dasgupta, I., Dutt, N., Patil, B.L., Jeske, H., 2006. Biodiversity and recombination of cassava-infecting begomoviruses from southern India. Arch. Virol. 151, 55–69.

Sambrook, J., Russell, R., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Saunders, K., Wege, C., Karuppannan, V., Jeske, H., Stanley, J., 2001. The distinct disease phenotypes of the common and yellow vein strains of Tomato golden mosaic virus are determined by nucleotide differences in the 3′-terminal region of the gene encoding the movement protein. J. Gen. Virol. 82, 45–51.

Saunders, K., Salim, N., Mali, V.R., Malathi, V.G., Briddon, R., Markham, P.G., Stanley, J., 2002. Characterisation of Sri Lankan cassava mosaic virus and Indian cassava mosaic virus: evidence for acquisition of a DNA B component by a monopartite begomovirus. Virology 293, 63–74.

Stanley, J., Gay, M.R., 1983. Nucleotide sequence of cassava latent virus DNA. Nature 301, 260–262.

Stanley, J., Bisaro, D.M., Briddon, R.W., Brown, J.K., Fauquet, C.M., Harrison, B.D., Rybicki, E.P., Stenger, D.C., 2005. Geminiviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A (Eds.), Virus Taxonomy. VIIIth Report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press, London, pp. 301–326.

Sudarshana, M.R., Wang, H.L., Lucas, W.J., Gilbertson, R.L., 1998. Dynamics of bean dwarf mosaic geminivirus cell-to-cell and long-distance movement in *Phaseolus vulgaris* revealed, using the green fluorescent protein. Mol. Plant-Microb. Interact. 11, 277–291.

Sunter, G., Bisaro, D.M., 1997. Regulation of a geminivirus coat protein promoter by AL2 protein (TrAP): evidence for activation and derepression mechanisms. Virology 232, 269–280.

Unseld, S., Höhnle, M., Ringel, M., Frischmuth, T., 2001. Subcellular targeting of the coat protein of African cassava mosaic geminivirus. Virology 286, 373–383.

van Wezel, R., Dong, X., Liu, H., Tien, P., Stanley, J., Hong, Y., 2002. Mutation of three cysteine residues in Tomato yellow leaf curl virus-China C2 protein causes dysfunction in pathogenesis and posttranscriptional gene-silencing suppression. Mol. Plant-Microb. Interact. 15, 203–208.

van Wezel, R., Liu, H., Wu, Z., Stanley, J., Hong, Y., 2003. Contribution of the zinc finger to zinc and DNA binding by a suppressor of posttranscriptional gene silencing. J. Virol. 77, 696–700.

Vanitharani, R., Chellappan, P., Pita, J.S., Fauquet, C.M., 2004. Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. J. Virol. 78, 9487–9498.

Voinnet, O., Pinto, Y.M., Baulcombe, D.C., 1999. Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. Proc. Natl. Acad. Sci. U. S. A. 96, 14147–14152.

Waigmann, E., Ueki, S., Trutnyeva, K., Citovsky, V., 2004. The ins and outs of nondestructive cell-to-cell and systemic movement of plant viruses. Crit. Rev. Plant Sci. 23, 195–250.

Wartig, L., Kheyr-Pour, A., Noris, E., Dekouchkovsky, F., Jouanneau, F., Gronenborn, B., Jupin, I., 1997. Genetic analysis of the monopartite tomato yellow leaf curl geminivirus: roles of V1, V2 and C2 ORFs in viral pathogenesis. Virology 228, 132–140.

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

Zhang, S.C., Wege, C., Jeske, H., 2001. 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. Virology 290, 249–260.

Zhang, S.C., Ghosh, R., Jeske, H., 2002. Subcellular targeting domains of Abutilon mosaic geminivirus movement protein BC1. Arch. Virol. 147, 2349–2363.