Expression dynamics and ultrastructural localization of epitope-tagged Abutilon mosaic virus nuclear shuttle and movement proteins in *Nicotiana benthamiana* cells

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**A B S T R A C T**

The geminivirus Abutilon mosaic virus (AbMV) encodes two proteins which are essential for viral spread within plants. The nuclear shuttle protein (NSP) transfers viral DNA between the nucleus and cytoplasm, whereas the movement protein (MP) facilitates transport between cells through plasmodesmata and long-distance via phloem. A cell-free assay system for epitope-tagged NSP and MP in plants yielded unprecedented amounts of both proteins. Western blots revealed extensive posttranslational modification and truncation for MP, but not for NSP. Ultrastructural examination of *Nicotiana benthamiana* tissues showed characteristic nucleopathic alterations, including fibrillar rings, when epitope-tagged NSP and MP were simultaneously expressed in leaves locally infected with an AbMV DNA A in which the coat protein gene was replaced by a green fluorescent protein encoding gene. Immunogold labelling localized NSP in the nucleoplasm and in the fibrillar rings. MP appeared at the cell periphery, probably the plasma membrane, and plasmodesmata.

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**Introduction**

The plant geminivirus family (*Stanley et al., 2005*) consists of viruses with either one or two genomic components of circular single-stranded (ss) DNA within their twinned icosahedral particles (*Böttcher et al., 2004; Zhang et al., 2001*). Viral DNAs are replicated via double-stranded intermediates within the nuclei of host plant cells (*Jeske, 2007, 2009*). Consequently, the viral DNA has to cross two barriers for systemic spread, the nuclear envelope and the plasma membrane/cell wall. For bipartite begomoviruses, two open reading frames (ORFs) located on the DNA B component encode proteins that manage the tasks: ORF BV1 (syn. BR1) encodes a nuclear shuttle protein (NSP) and ORF BC1 (syn. BL1) the movement protein (MP) (*Gafni and Epel, 2002; Jeske, 2009; Rojas et al., 2005; Wege, 2007*). Moreover, both proteins have an impact on viral pathogenicity (*Jeske, 2009; Rojas et al., 2005; Zhou et al., 2007*). For bipartite begomoviruses, the coat protein (CP) is not essential for systemic infection, suggesting a transport complex different from virions (*Jeske, 2009; Rojas et al., 2005*). Nevertheless, CP was able to complement defective NSP mutants, and is therefore regarded as a redundant element in viral movement (*Qin et al., 1998*).

Several cellular localization studies have identified NSP of different begomoviruses within plant nuclei and MP at the cell periphery (*Pascal et al., 1994; Sanderfoot and Lazarowicz, 1995; Zhang et al., 2002, 2001*). Cell fractionation assays supported these observations, as they revealed MP in the cell wall- or plasma membrane-enriched fractions, whereas NSP was found in different fractions than MP (*Pascal et al., 1993; von Arnim et al., 1993; Ward et al., 1997*). Interestingly, the subcellular distribution of MP changed during systemic infection by *Squash leaf curl virus* (SLCV) in pumpkin leaf tissues (*Ward et al., 1997*). In the early phase of infection, characterized by the onset of symptom development and high MP concentrations, the protein occurred mainly in the endoplasmic reticulum (ER) comprising cell fractions. As symptoms progressed and the SLCV-infected leaf matured the MP content in the ER-containing fractions decreased and increased in the plasma membrane-enriched fractions. Microinjection of fluorescently labelled Bean dwarf mosaic virus (BDMV) MP into plant mesophyll cells showed protein movement between cells and size-exclusion limit (SEL) modification of plasmodesmata (*Nourety et al., 1994*). In contrast, NSP failed to be transferred between cells and to alter the SEL under the same assay conditions, but shuttled viral DNA from the nucleus into the cytoplasm. Although it is well established that NSP facilitates trafficking of viral DNA into and out of the nucleus, and MP serves as a membrane adaptor and mediates cell-to-cell transfer via plasmodesmata as well as long-distance spread through the phloem (*Jeske, 2009; Krichevsky et al., 2006; Rojas et al., 2005*), the details of how both proteins coordinate viral DNA transport of bipartite begomoviruses are still under debate. The “couple-skiing” model suggests that MP binds the NSP–DNA complex at the cytoplasmic side of plasma membranes or mesosomal vesicles, and transfers the nucleoprotein complex into the next cell either along the plasma membrane or via the ER that spans the plasmodesmata (*Aberle et al., 2002, 2001*).
2002; Frischmuth et al., 2004, 2007; Hehnle et al., 2004; Pascal et al., 1994; Sanderson and Lazarowitz, 1995; Zhang et al., 2002, 2001). In contrast, the "relay race" model predicts that after NSP-mediated export viral DNA is taken over by MP, which then transports it into the adjacent cell (Noueiry et al., 1994; Rojas et al., 2005, 1998). Our own data, obtained for Abutin mosaic virus (AbMV), are more compatible with the "couple-skating" model (Aberle et al., 2002; Frischmuth et al., 2004, 2007; Hehnle et al., 2004; Jeske, 2009; Zhang et al., 2002, 2001). AbMV NSP fused with green fluorescent protein (GFP) exhibited an exclusive nuclear localization when singly expressed in plant cells, whereas GFP-tagged MP was associated with the cell periphery and appeared also peri-nuclear. After co-expression, NSP was redirected to the cell periphery and to adjacent cells of plant sink tissues indicating their transport capability even in mesophyll cells in spite of the phloem-limitation of AbMV during systemic infection (Horns and Jeske, 1991; Wege et al., 2001; Zhang et al., 2001). The small number of phloem-limited infection sites and the transient expression during viral spread caused low NSP and MP concentrations within AbMV-infected plants and has so far prevented a detailed study of their functional role during systemic movement (for discussion see Kleinow et al., 2008, 2009). Recently, we identified proteins so as to reduce their pathogenic side effects. We analyzed the transient and inducible overexpression assay for epitope-tagged proteins so as to reduce the frequency of paracrine side effects. We analyzed the induction profiles and turn-over rates of singly and co-expressed NSP and MP by western blot analysis and localized both epitope-tagged proteins at the ultrastructural level in N. benthamiana cells during local co-infection with an AbMV DNA A replicon in which the CP gene was replaced by GFP. The results confirmed the association of MP with plasma membranes and plasmodesmata and showed for the first time an association of NSP with fibrillar rings which had previously been observed for other geminiviruses and noted as diagnostic nucleoplastic alterations.

Results

Expression dynamics of epitope-tagged AbMV NSP and MP in plant cells

Manifold trials to ectopically overexpress AbMV NSP and MP in plant tissues under the control of the 35S promoter, either in Arabidopsis thaliana, cell suspension culture or in transgenic Nicotiana benthamiana plants, have failed, presumably because of pathogenic side effects from both proteins (data not shown). Although tissue cultures and plants grew well under antibiotic selection and harboured the expression cassette, western blots revealed only negligible amounts of NSP or MP, indicating possible silencing of the transgene (data not shown). This problem has been solved by the use of estradiol-inducible constructs from which NSP or MP were transiently expressed after agro-infiltration of N. benthamiana leaves (Fig. 1A). We expressed MP and NSP fused to intron-disrupted epitope tags (c-Myc for MP and hemagglutinin [HA] for NSP, Fig. 1A), a precaution which was necessary to ensure the exclusive detection of plant-originated proteins. To maximize their yield the target proteins of comparative samples were co-expressed with the silencing suppressor p19 from Cymbidium ringspot virus (Lakatos et al., 2004). Protein levels were analyzed by western blots during a time course experiment (Fig. 2). In control experiments, blots were stripped after epitope-specific immunodetection and re-probed by the respective anti-AbMV MP or NSP antisera. Since specific signals thus obtained co-localized, the identity of c-Myc-MP or HA-NSP bands was unequivocally confirmed (data not shown). For western blot analyses of samples obtained from combined expression of c-Myc-MP and HA-NSP, the membrane was first probed for c-Myc-MP and after stripping, re-probed with antibodies specific for HA-NSP. Buffer-infiltrated leaves were used as mock-treated controls. The anti-c-Myc antibody as well as the anti-AbMV NSP antisera cross reacted with plant proteins, which can be differentiated from the c-Myc-MP or HA-NSP bands by comparison with the mock-inoculated reference samples (Figs. 2C and D, lane M). Unprecedented levels of c-Myc-MP- and HA-NSP-specific signals were obtained using either epitope-specific antibodies (Figs. 2A–C) or anti-AbMV NSP antisemur (Fig. 2D) upon controlled transient expression in planta, which were

Fig. 1. Schematic representation of epitope-tagged AbMV MP and NSP expression constructs and the AbMV DNA A-based replicon. (A) Fusion of the MP gene with an intron-disrupted coding sequence of the c-Myc epitope or of the NSP gene with an intron-disrupted coding sequence of the HA epitope in the estradiol-inducible expression vector pMDC7 (Curts and Grossniklaus, 2003; Zuo et al., 2000). Triangles represent introns present in the epitope tags. Orientations of the left (LB) and right (RB) T-DNA borders are indicated. P35S2, synthetic promoter controlling XVE expression; XVE, chimeric transcription factor consisting of LexA DNA binding domain, VP16 transcription activation domain and regulatory domain of human estrogen receptor; Tps, pea rbcS-3A terminator; Hygr, hygromycin resistance marker, O2mar, 8 copies of LexA operator sequence coupled to 35S minimal promoter (P35S2), attB1/attB2; DNA recombination sequences, Tps2, pea rbcS-3A terminator. (B) In a partial dimer of AbMV DNA A the CP ORF was replaced by the mGFP4 coding sequence (Krenz, 2007). The resulting cassette was transferred into BInPlus (van Engelen et al., 1995) to yield the agroinfectious binary vector pBIn-TR224 (DNA A/DAP/GFP). Upon delivery into plant cells a stable replicon 52 bp larger than AbMV DNA A will be released. In planta mGFP4 expression is driven by the AV1 (syn. AR1) promoter. Assigned gene functions of indicated ORFs: AC1 (syn. AL1): replication-associated protein, Rep; AC2 (syn. AL2): transcriptional activator protein, TrAP; AC3 (syn. AL3): replication enhancer, Ren; AV1: coat protein, CP; CR: common region. Restriction sites used for cloning procedure: B: BamHI; E: EcoRI; H: HindIII blunted by fill-in reaction; S: SacI; X: XmaI.
boosted by the presence of p19. A distinct c-Myc-MP-specific signal in the range of the calculated molecular mass of the fusion protein (35.6 kDa) and specific bands with retarded migration behaviour were detectable (Figs. 2A and C, bracket). The full-length c-Myc-MP formed a similar multitude of bands (Figs. 2A and C) as previously found for MP derived from AbMV-infected plants (Kleinow et al., 2008; Wege and Pohl, 2007; Zhang et al., 2001) or from yeast (Aberle et al., 2002; Frischmuth et al., 2004; Kleinow et al., 2008, 2009). The MP amounts observed consistently for the treated samples (Figs. 3B, C) or even earlier (data not shown). The decline is early as 6 hpei, its level increased until 48 hpei and declined thereafter (Figs. 2B and D, arrow). Large amounts of truncated c-Myc-MP along with stable HA-NSP have been observed in four independent co-expression experiments (data not shown). HA-NSP was detected as early as 6 hpei, its level increased until 48 hpei and declined thereafter (Figs. 2B and D) or even earlier (data not shown). The decline is diminished significantly in the presence of p19, indicating that post-transcriptional gene silencing (PTGS) may control the levels of HA-NSP. Such a regulated appearance of HA-NSP was found reproducibly in single (five experiments) and combined expression with c-Myc-MP (four experiments) (data not shown).

The reliably high amounts of epitope-tagged AbMV MP and NSP in N. benthamiana, presented here for the first time, encouraged us to characterize their fate directly in planta and provided thereby the possibility to combine the overexpressed proteins with a viral DNA template.

Ultrastructural localization of epitope-tagged NSP and MP in plant cells

We investigated the subcellular localization of HA-NSP and c-Myc-MP in leaf tissues co-expressing both proteins and replicating a modified AbMV DNA A, by electron microscopy of ultrathin sections using immunogold labelling. The HA-NSP and the c-Myc-MP expression constructs along with a binary vector harbouring a partial dimer of AbMV DNA A in which the CP gene was replaced by GFP [Fig. 1B, pBK-TR224(DNA ΔCP:GFP), Krenz, 2007] were delivered by simultaneous agro-infiltration into leaves of N. benthamiana. In plant cells, an autonomous circular DNA ΔCP:GFP replicon can thus be generated and the DNA progeny may associate with the test proteins in the absence of CP, which would facilitate the discrimination of ultrastructural complexes. GFP-positive leaf tissues at 13 hpei were prepared for electron microscopy and buffer-infiltrated leaves served as mock control. Resin blocks of four independent samples for mock (example Fig. 3A) and treated (example Figs. 3B–C) leaves were analyzed and the structure of ultrathin sections was compared after initial analysis of semi-thin sections by light microscopy and toluidine blue staining. Several alterations of the nuclear architecture were observed consistently for the treated samples (Figs. 3B–G). Nuclei...
Fig. 3. Immunogold labelling using anti-HA monoclonal antibodies of overexpressed AbMV HA-NSP in plant cells. Electron micrographs of nuclei were taken from resin-embedded N. benthamiana leaf sections either after mock-treatment (A) or after co-infiltration with agrobacteria harbouring pBK-TR224(DNA AΔCP:GFP), which released a replicating AbMV DNA AΔCP:GFP circle in planta, and HA-NSP and c-Myc-MP constructs for inducible expression (B–G). (F) and (G) represent close-ups of (E). Arrows mark cytoplasmic invaginations into the nucleus. No: nucleolus, G: granular, and F: fibrillar region of nucleoli, Ch: peripheral plant chromatin, Fb: fibrillar bodies. Sizes of scale bars 500 nm.
frequently exhibited cytoplasmic invaginations associated with condensation of plant heterochromatin close to the nuclear envelope (Figs. 3C and E, Ch). Nucleoli (Fig. 3, No) were enlarged and sometimes segregated into fibrillar and granular compartments (Figs. 3B and D) as previously observed for plants systemically infected with several begomoviruses (Adjeare and Coutts, 1982; Kim and Flores, 1979; Kim and Fulton, 1984; Kim et al., 1978). Additionally, fibrillar bodies (Figs. 3B and E–G, Fb) either solid or ring-shaped with various sizes scattered randomly in the nucleoplasm without association to the nucleolus were present. They were found exclusively in those nuclei which also exhibited peripheral heterochromatin distribution and cytoplasmic invaginations. A detailed inspection of the fibrillar bodies in serial sections revealed that they likely represent hollow spheres or shells (data not shown). No cellular abnormalities were observed in other organelles, such as chloroplasts, or in the cytoplasm under these conditions, confirming that the fixation and embedding procedure was suitable.

Before being able to evaluate the immuno-labelling, we had to solve the problem of non-specific binding of the anti-HA and anti-c-Myc antibodies if applied in too high a concentration. To this end we carefully optimized the proper antibody dilutions during several rounds of experiments until we obtained a signal to noise ratio which allowed a reliable localization of HA-NSP as well as c-Myc-MP in comparison to mock-treated samples. In order to assess the validity of immunogold labelling, micrographs of nuclei were taken randomly and their enlarged images after immunogold labelling were compared to mock-treated samples. In order to assess the validity of immunogold labelling, micrographs of nuclei were taken randomly and their enlarged images thereafter (Table 1). In comparison to nuclei of mock controls, the majority of nuclei from the HA-NSP/c-Myc-MP/DNA AΔCP:GFP plant samples exhibited a significantly higher number of gold grains. This labelling was homogeneously distributed over the nucleoplasm and nucleolus (Figs. 3C–G), sometimes more concentrated within the nucleolus (Figs. 3B, D) or over the fibrillar rings (Figs. 3F, G). No significant labelling was observed outside the nuclei, even though we inspected the thin sections extensively in the hope of identifying NSP complexes at the plasma membrane.

Although we tested several dilutions of the anti-c-Myc antibody for detection of MP, we obtained a similar immunogold labelling of nuclei for mock and treated samples (data not shown) indicative for a cross reactivity towards a nuclear localized plant protein, presumably a $\alpha$-Myc epitope containing transcription factor. Therefore, no final conclusion is possible about a nuclear localization of c-Myc-MP. In contrast, a differential and significant c-Myc-MP-specific gold labelling was detected in close association with the cellular margin (Figs. 3C–G) and secondary plasmodesmata (Fig. 4I). Enhanced labelling was particularly evident at the cell periphery in close proximity to nuclei with altered architecture and fibrillar bodies in bundle cell areas. The exact position of the immuno-labelling is difficult to assess. First of all, the combination of primary and secondary antibody may place the gold grain up to 20 nm away from the binding site. Second, a plasma membrane which is cut perpendicular to its plane reveals relatively few grains (e.g. Fig. 4I). For plasma membranes which are sectioned in a more parallel orientation, and which thus present more binding sites for the antibody on the surface (e.g. Figs. 3D, G), the labelling may be misinterpreted as lying over the cell wall for spatial-structural reasons.

In summary, the results showed the first high resolution view on the differential accumulation of geminiviral NSP and MP in the absence of CP during local infection. We identified the nuclear fibrillar rings as NSP containing, and an association of MP with the cell periphery, presumably at the plasma membranes, and plasmodesmata as discussed below.

### Discussion

The inducible overexpression of epitope-tagged AbMV MP and NSP enabled us for the first time to compare the expression dynamics of both proteins in planta. The presence of the silencing suppressor p19 enhanced HA-NSP and c-Myc-MP levels significantly and suggested a general impact of silencing on their expression. Whereas c-Myc-MP accumulated continuously during the time course investigated, HA-NSP levels increased transiently despite the presence of the silencing suppressor. It remains to be determined which mechanism is causing this interesting difference between the c-Myc-MP and HA-NSP accumulation profile, but silencing seems to play not a decisive role in this particular aspect. Possibly the HA-NSP possess a shorter half-life than c-Myc-MP within plant cells. The detection of prominent c-Myc-MP-specific bands with reduced mobility, which accumulated with time of expression, reflects most likely phosphorylation, but possibly also other unidentified posttranslational modifications, as investigated and discussed earlier (Kleinow et al., 2008, 2009). In contrast, HA-NSP appeared as a single band, suggesting that it is less targeted by posttranslational modifications than MP under these expression conditions, or the modifications may have no influence on its migration behaviour. Epitope-tagged MP-specific bands with lower apparent molecular mass accumulated reproducibly with time. It would be interesting to determine whether they are simply caused by overexpression or reflect a specific cleavage of MP. Following enrichment of microsomes from AbMV-infected plants, we observed truncated MP variants in western blot analysis, too (Kleinow et al., 2008). Other studies on plant-derived MP found as well multiple bands with retarded migration behaviour and truncated variants in western blots (Duan et al., 1997a, 1997b; Pascal et al., 1993; von Arnim et al., 1993). Therefore, we hypothesize that MPs do not only undergo several posttranslational modifications, but possibly also an intrinsic processing.

In locally AbMV DNA AΔCP:GFP-infected leaf tissues co-expressing HA-NSP and c-Myc-MP, several changes in the nuclear architecture occurred. Most strikingly were the emergence of fibrillar rings in nuclei, enlarged and segregated nucleoli as well as the redistribution of the plant chromatin to the nuclear margin, which is a characteristic phenomenon for cells arrested in early mitosis (Bass et al., 2000). These findings correspond to typical alterations described for nuclei of various dicotyledonous host plants which had been systemically infected with different geminiviruses (Adjeare and Coutts, 1982; Bass et al., 2000; Kim et al., 1986; Kim and Flores, 1979; Kim and Fulton, 1984; Kim et al., 1978; Lastra and Gil, 1981; Roberts, 1989; Rushing et al., 1987; Thongmeeark et al., 1981). Hypertrophy of the nucleus and nucleolus, segregation of nucleolar compartments into discrete granular and fibrillar regions, emergence of fibrillar rings varying in number and sizes have been frequently found associated with the formation of nuclear inclusion bodies containing virus-like gemini particles. Cytochemical studies revealed that the fibrillar rings contained DNA and protein (Kim et al., 1986, 1978). As virus particles occurred first near the nucleoli or the fibrillar bodies, they were interpreted as sites of DNA synthesis and virion assembly. In our experimental set-up leaf tissues were infected with an AbMV DNA A

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**Table 1**

Number of gold grains counted within nuclei after immunogold labelling using anti-HA antibody of electron microscopic preparations from N. benthamiana either mock-treated or expressing HA-NSP and c-Myc-MP in leaf tissues locally infected with AbMV DNA AΔCP:GFP.

<table>
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<tr>
<th>No. gold grains</th>
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<td>0–20</td>
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<td>21–30</td>
<td>7</td>
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<td>31–40</td>
<td>0</td>
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<tr>
<td>41–50</td>
<td>0</td>
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<tr>
<td>51–60</td>
<td>0</td>
</tr>
<tr>
<td>61–200</td>
<td>0</td>
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<tr>
<td>Sum of nuclei</td>
<td>28</td>
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Fig. 4. Immunogold labelling using anti-c-Myc monoclonal antibodies of overexpressed AbMV c-Myc-MP in plant cells. Plant samples either mock-treated (A, B and H) or co-infiltrated (C–G and I) were prepared as described in Fig. 3. (D) represents an image at higher magnification of the marked cell wall area (asterisks) in (C). Arrows highlight gold grains associated with the cell periphery (C) or plasmodesmata (I). CW: cell wall, Pd: plasmodesmata. Sizes of scale bars 500 nm.
lacking a functional CP precluding particle production. Thus, the development of fibrillar bodies seems to be independent from virion formation and likely represents nuclei containing replicating viral DNA complexed with NSP. In addition to the previously described typical geminivirus induced nucleopathic alterations, we frequently observed leaf samples with intense cytoplasmic invaginations into their nuclei. They substantially increase the nuclear surface area and may thereby play a role in efficient trafficking between nucleus and cytoplasm (Collings et al., 2000). Previous electron microscopic studies on AbMV – and Sida micrantha mosaic virus – (SimMV, Jovel et al., 2004), at that time thought to be a Brazilian strain of AbMV infected host plants showed several cytopathological alterations (Abouzid et al., 1988; Jeske, 1986; Jeske et al., 1977; Jeske and Schuchalter-Eicke, 1984; Jeske and Werz, 1980a, 1980b; Schuchalter-Eicke and Jeske, 1983). Geminivirus-like twin particles accumulated in nuclear paracrystalline tubes or amorphous inclusions. They reacted with anti-SimMV CP antibodies (Abouzid et al., 1988) and were, thus, identified as virions. No such structures were found in the current study confirming that they solely rely on CP and are not produced with NSP alone. For SimMV, but not AbMV, nuclear inclusions with fibrillar material appeared concomitantly with geminivirus infection and were not decorated with anti-SimMV CP antibodies (Abouzid et al., 1988). In size and stainability, the fibrillar material was similar to the fibrillar bodies in the current study. Structures resembling fibrillar inclusions were also found in plastids of SimMV-infected Malva parviflora plants (Jeske, 1986; Jeske and Schuchalter-Eicke, 1984; Jeske and Werz, 1980a, 1980b) and were at that time thought to represent packaged viral DNA in plastids (Gröning et al., 1987, 1990). However, we could never reliably detect these structures with CP-specific antibodies (Abouzid et al. (1988) and unpublished data) and they were absent in the current study. Usually, a systemic geminivirus infection leads to dramatic changes in plastid ultrastructure dependent on light intensity, diurnal, and seasonal conditions (Jeske, 1986; Jeske and Werz, 1978; Schuchalter-Eicke and Jeske, 1983). These cytopathic effects, as observed in mesophyll cells, were interpreted as an indirect result of blocked carbohydrate translocation in the virus-infected phloem (Jeske and Werz, 1978). In agreement with this idea, no direct effect on the chloroplast structure was detected here in the virus-treated samples. In a previous study, SimMV virions in the cytoplasm of young differentiating phloem cells were decorated by an antiserum raised against CP (Abouzid et al., 1988), presumably representing virus particles being transported from or into the nucleus. No such particles were detected in the current study.

Immunogold labelling revealed localization of HA-NSP in the nucleus and of c-Myc-MP close to cell walls, presumably at plasma membranes in plant cells. Moreover, we showed for the first time an association of a begomoviral MP with secondary plasmodesmata at the ultrastructural level. These findings are in agreement with previous studies in which GFP-tagged AbMV MP and NSP were transiently expressed in plant cells after biolistic bombardment of DNA. Therein, NSP localized to the nucleus, whereas MP associated with the cell periphery and around nuclei (Zhang et al., 2002, 2001). However, upon co-expression of both proteins in sink tissues, GFP-NSP was redirected to the cell periphery and to adjacent cells (Zhang et al., 2001), a translocation which could not be detected in the present study. Several explanations are conceivable for this failure: first, a small subpopulation of NSP outside the nucleus may have been missed simply due to technical limitations. Most shuttling proteins at equilibrium are predominantly nuclear, having small cytoplasmic pools that may be not detected by conventional immunological techniques (Laskey and Dingwall, 1993; Lazarowitz and Beachy, 1999; Schmidt-Zachmann et al., 1993). Second, we may not have found the proper cell-to-cell transport-competent cells in the more labour intensive electron microscopic work compared to fluorescence microscopy (see discussion in Zhang et al. (2001) for tissue-dependent transport). Third, the modifications by the chosen tags may have prevented proper transport complex formation. Fourth, not all observed cells may have expressed both target proteins at the same time, a prerequisite which cannot be easily controlled for electron microscopic analysis. Fifth, the transport may be too efficient and rapid in this assay to be trapped in an intermediate state. Moreover, ultrathin-sectioning does not allow the inspection of large membrane areas in the cross-section, thus missing small amounts of NSP targeted to the plasma membrane just for structural reasons. We could demonstrate this problem using freeze-fracture immuno-labelling of NSP co-expressed with MP in fission yeast cells (Aberle et al., 2002; Frischmuth et al., 2007), a technique which is better suited for examining membrane-bound proteins.

Our ultrastructural data for the subcellular distribution of AbMV NSP agree well with the few other studies of geminivirus movement-associated proteins that have been conducted. Immunogold labelling localized SLCV NSP in nuclei of systemically infected pumpkin plants (Pascal et al., 1994). Although re-localization of SLCV NSP to the cell periphery by SLCV MP had been observed in protoplasts and insect cells (Sanderfoot et al., 1996; Sanderfoot and Lazarowitz, 1995, 1996), the authors were also unable to track this redirection by immunogold labelling on ultrathin sections (Pascal et al., 1994). In contrast with AbMV NSP, SLCV NSP was preferentially found within the nucleus of infected nuclei and no fibrillar bodies were reported. Using the same technique, SLCV MP was localized at ER-derived tubules, but only in specialized tissues of the vascular bundle. Interestingly, these tubules extended up to and across the cell wall in procambial cells (Wood et al., 1997). No such structures could be observed in our samples of inoculated leaves and therefore they are possibly present only during systemic infection in vascular tissues. The difference may also be due to the different viruses used. One further electron microscopic analysis has been performed on the movement protein of the monopartite Maize streak virus (Dickinson et al., 1996). For mastreviruses, the V1 protein facilitates cell-to-cell transport (Boulton et al., 1993; for review see Jeske, 2009; Rothenstein et al., 2007). As for AbMV MP, MSV V1 was localized at the plasma membrane and plasmodesmata in maize leaf tissues.

In summary, the controlled overexpression of epitope-tagged AbMV NSP and MP provided several novel insights into the dynamics and subcellular localization of geminiviral transport components. They showed a remarkable multitude of MP forms at the highest resolution and signal intensity so far possible and allowed us to assign ultrastructural inclusion bodies, appearing as fibrillar rings, to higher order NSP complexes. In addition, the results of the few other ultrastructural investigations are further confirmed concerning the dichotomy of NSP and MP localization within the nucleus and at plasma membranes, respectively.

Materials and methods

Cloning procedure

For controlled expression in planta, the full-length AbMV NSP coding region was subcloned using BamHI restriction sites from a pGEM-T construct (Frischmuth et al., 2004) into the pPCV812-Menchu binary vector (Ferrando et al., 2000). The resulting fusion of the NSP ORF with the intron-tagged coding sequence of the Ha epitope (pPCV812-Menchu) was confirmed by restriction endonuclease digestion and sequencing. To generate a GATEWAY entry clone (Invitrogen, Carlsbad, CA) carrying the HA-NSP fragment, pPCV812-Menchu-NSP was linearized with HindIII at the 5′ end of the epitope-tag coding region and overhangs were filled-in with Klenow DNA polymerase. The HA-NSP fragment was released on its 3′ end by cutting with EcoRI and inserted into the XmnI and EcoRI sites of pENTR111 (Invitrogen). The entry clone was confirmed by restriction analysis, and the DNA fragment was recombined into a GATEWAY compatible version of the pER8 binary vector (pMDC7, Curtis and Grossniklaus, 2003), from which HA-tagged NSP can be expressed in
an estradiol-inducible manner (Zuo et al., 2000, Fig. 1A). The final binary vectors were verified by restriction analysis and sequencing. Other constructs used in this study were described previously (Kleinow et al., 2009; Krenz, 2007).

**Transient expression of AbMV MP and NSP in N. benthamiana**

Binary vectors were transformed into Agrobacterium tumefaciens GV3101 (pMP90RK) strain (Koncz et al., 1994) using a chemical method, and the integrity of the introduced expression constructs was confirmed by PCR. Recombinant agrobacteria were infiltrated either singly or in combination into leaves of three-week-old N. benthamiana Domin plants according to Voinnet et al. (2003). Each experimental set was performed in parallel with or without co-infiltration of agrobacteria harbouring a 35S promoter-driven expression cassette for the silencing suppressor p19 (Lakatos et al., 2004). 48 h after infiltration, expression was induced by applying estradiol solution (10 μM estradiol in tap water with 0.005% [v/v] SILWET L-77) onto leaves. Leaf material was harvested and shock-frozen in liquid nitrogen at different hpe (compare Fig. 2). For protein extraction, plant tissues were homogenized in SDS-PAGE loading buffer (2% SDS, 6.5% glycerol, 62.5 mM Tris–HCl, 5% β-mercaptoethanol, 0.002% bromophenol blue; pH 6.8) and boiled for 5 min at 95 °C. Supernatants recovered after centrifugation (10 min, room temperature, 13,000 g) were directly subjected to SDS-PAGE.

**SDS-PAGE and western blotting**

Protein samples were separated by 12.5% SDS-PAGE and semidry-blotted onto nitrocellulose membranes as described (Kleinow et al., 2008). Blots were incubated in blocking buffer (TBST [137 mM NaCl, 0.1% (v/v) Tween 20, 20 mM Tris–HCl pH 7.6] containing 5% [w/v] non-fat dry milk powder) for 1 h at room temperature, and then with either rabbit anti-AbMV NSP polyclonal antiserum (Wege and Pohl, 2007), rat anti-HA (Roche, Mannheim, Germany) or mouse anti-c-Myc monoclonal antibodies (Sigma, Taufkirchen, Germany) diluted in blocking buffer (1: 1,000 anti-AbMV NSP; 1:2,000 anti-HA; 1:5,000 anti-c-Myc) overnight at 4 °C. After washing once for 15 min and 4 times for 5 min with TBST, membranes were incubated at room temperature for 1 h either with phosphate-conjugated (1: 2,500 goat anti-rabbit, Biotrend, Cologne, Germany) or peroxidase-conjugated secondary antibodies (1:10,000 goat anti-mouse, Biotrend or goat anti-rat, Sigma) diluted in blocking buffer. Washing steps were repeated as described above. Peroxidase-conjugated antibodies were detected by the enhanced chemiluminescence method (Visualizer Spray & Glow ECL detection system, Upstate/Millipore, Eschborn, Germany) and exposure to X-ray films. Peroxidase-conjugated antibodies were visualized by a colorimetric method as described (Wege and Pohl, 2007).

**Electron microscopy**

HA-NSP and the c-Myc-MP estradiol-inducible expression constructs were agro-infiltrated along with the binary vector pBKK-TR224 (DNA AACP-GFP) (Krenz, 2007), which releases an autonomously replicating AbMV DNA A circle with the CP gene replaced by an ORF encoding GFP in plants (Fig. 1B). Expression of epitope-tagged AbMV NSP and MP was induced as stated above. Leaves were inspected for GFP fluorescence using a hand-held long-wave UV lamp (4 W) at 13 hpe. GFP-positive leaf areas were subjected to tissue preparation for electron microscopy according to Abouzid et al. (1988). Buffer-infiltrated leaves served as a mock-treated reference and were processed in parallel. Detection of epitope-tagged AbMV NSP or MP in ultrathin sections of epoxy resin-embedded leaf tissues by immunogold labelling was essentially carried out as described (Abouzid et al., 1988) with the following modifications: (i) incubation with primary monoclonal anti-epitope antibodies (rat anti-HA [Roche] dilution 1:150; mouse anti-c-Myc [Sigma] dilution 1:10,000) was performed at 4 °C over night and (ii) samples were incubated with secondary gold-tagged antibodies (goat anti-rat IgG 12 nm colloidal gold [Dianova, Hamburg, Germany] and goat anti-mouse IgG 10 nm colloidal gold [Sigma], each diluted 1:20) for 1 h at room temperature. After immunogold labelling, samples were fixed (1% glutaraldehyde in PBS for 5 min) and washed 4 times for 1 min as well as 2 times for 10 min with water. Following air-drying, the sections were stained with saturated uranyl acetate in 70% methanol for 5 min and lead citrate for 10 min (Venable and Coggleshall, 1965). Transmission electron microscopy was performed as described (Jeske et al., 2001).

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**References**


