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A plastid-targeted heat shock cognate 70 kDa protein interacts with the *Abutilon* mosaic virus movement protein

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ABSTRACT

The movement protein (MP) of bipartite geminiviruses facilitates cell-to-cell as well as long-distance transport within plants and influences viral pathogenicity. Yeast two-hybrid assays identified a chaperone, the nuclear-encoded and plastid-targeted heat shock cognate 70 kDa protein (cpHSC70-1) of *Arabidopsis thaliana*, as a potential binding partner for the *Abutilon* mosaic virus (AbMV) MP. *In planta*, bimolecular fluorescence complementation (BiFC) analysis showed cpHSC70-1/MP complexes and MP homooligomers at the cell periphery and co-localized with chloroplasts. BiFC revealed cpHSC70-1 oligomers associated with chloroplasts, but also distributed at the cellular margin and in filaments arising from plastids reminiscent of stromules. Silencing the cpHSC70 gene of *Nicotiana benthamiana* using an AbMV DNA A-derived gene silencing vector induced minute white leaf areas, which indicate an effect on chloroplast stability. Although AbMV DNA accumulated within chlorotic spots, a spatial restriction of these occurred, suggesting a functional relevance of the MP–chaperone interaction for viral transport and symptom induction.

Introduction

As chaperones, members of the heat shock protein 70 kDa (HSP70) class assist in various cellular processes such as folding of newly synthesized proteins, solubilising protein aggregates, disassembly of protein complexes, membrane translocation, and controlling the biological activity of regulatory proteins (Bukau et al., 2006; Kanzaki et al., 2003; Mayer and Bukau, 2005; Noel et al., 2007; Weibezahn et al., 2005). HSP70 transcript and protein levels may be controlled during plant development, enhanced by a variety of abiotic stresses and upregulated in response to infection with various plant viruses, including geminiviruses (Aparicio et al., 2005, and references therein; Brizard et al., 2006; Escaler et al., 2000a,b; Maule et al., 2000; Sung et al., 2001, and references therein; Swindell et al., 2007). Some HSP70 genes exhibit a low constitutive expression and are therefore called heat shock cognate protein (HSC) genes (Sung et al., 2001, and references therein; Swindell et al., 2007). In addition to the intracellular functions some HSP70s are involved in cell-to-cell transport of proteins. Two cytoplasmic *Cucurbita maxima* HSC70s were identified as non-cell-autonomous proteins (Aoki et al., 2002), and closteroviruses encode a protein homologous to HSP70 (HSP70h), that targeted to plasmodesmata and assembled into the virion tail of Beet yellows virus which is essential for virus transport (Alzhanova et al., 2007; Avisar et al., 2008 , and references therein). Identified interactions between plant HSP70-recruiting co-chaperones (DNAJ-like/HSP40 type) and movement proteins (MP) of various RNA viruses (Lu et al., 2009; Shimizu et al., 2009; Soellick et al., 2000) or the movement-associated coat protein of Potato virus Y (Hofius et al., 2007) suggest a role of these chaperones in viral spread and/or assembly.

The *Abutilon* mosaic virus (AbMV) belongs to the family *Geminiviridae* (Stanley et al., 2005). The replication of geminiviral single-stranded circular DNA genomes occurs in plant cell nuclei via double-stranded intermediates (Jeske, 2007, 2009). Hence for systemic spread, viral DNA has to cross two cellular barriers: the nuclear envelope and the plasmodesmata (Jeske, 2009; Krichevsky et al., 2006; Lucas, 2006; Rojas et al., 2005; Stanley et al., 2005; Waigmann et al., 2004). In the case of bipartite geminiviruses as AbMV, the two genes required for transport are located on the DNA B component. The open reading frame (ORF) BV1 (syn. BR1) encodes a nuclear shuttle protein (NSP), and ORF BC1 (syn. BL1) the movement protein (MP). Furthermore, both proteins affect viral pathogenicity (Jeske, 2009; Rojas et al., 2005; Zhou et al., 2007). Several studies have provided strong evidence that NSP is responsible for nuclear import and export of viral DNA, whereas MP serves as a membrane adapter and mediates cell-to-cell transport through plasmodesmata as well as long-distance transfer via the phloem (Jeske, 2009; Rojas et al., 2005). However, functional details of this process remain still to be determined. Localization studies of AbMV MP and NSP either singly or combined expressed in plant or yeast cells and analysis of *in vitro* assembled complexes of both proteins with DNA, suggest for AbMV a

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cell-to-cell transport according to the "couple-skating" model (Aberle et al., 2002; Frischmuth et al., 2004; Frischmuth et al., 2007; Hehnle et al., 2004; Kleinow et al., 2009b; Pascal et al., 1994; Sanderfoot and Lazarowitz, 1995; Zhang et al., 2001). In this model MP binds the NSP/DNA complex at the cytoplasmic face of plasma membranes or microsomal vesicles, and mediates its transfer into the adjacent cell either along the endoplasmic reticulum (ER) or the plasma membrane that spans plasmodesmata. Alternatively, the "relay race" model (Noueiry et al., 1994; Rojas et al., 1998) predicts that NSP passes viral DNA to MP which transports alone the DNA into the next cell (for recent review see, Jeske, 2009; Wege, 2007). Irrespective of the model, the geminiviral cell-to-cell transport represents a multistep mechanism in which the MP has to mediate a variety of different functions: (i) binding to DNA/NSP complexes or DNA (depending on the model), (ii) targeting to and modification of plasmodesmata, (iii) passage through plasmodesmata and (iv) release of the transport substrate after the transfer has been accomplished. During these processes, a series of interactions of MP with host proteins are expected, but none have been identified so far. Recently, we mapped three phosphorylation sites in AbMV MP, which play a role in symptom development and/or viral DNA accumulation (Kleinow et al., 2009a). Hence, kinases are most likely effecting MP functions and are candidates for MP-interacting plant factors.

In this study, we have screened an *Arabidopsis thaliana* yeast two-hybrid cDNA library using AbMV MP as a bait, and isolated a binding partner in the nuclear-encoded and plastid-targeted cpHSC70-1. In order to assess the relevance of this interaction *in planta*, bimolecular fluorescence complementation (BiFC) analyses were carried out in healthy and AbMV-infected plant tissues. The functional dependence of AbMV on the cpHSC70-1 protein for spread and/or symptom development in *Nicotiana benthamiana* was studied by help of virus-induced silencing of the cpHSC70 gene. In summary, the obtained data hint at an involvement of cpHSC70 chaperones in the AbMV life cycle.

Results

Identification of AbMV MP-interacting proteins of Arabidopsis

Previous deletion analysis of AbMV MP has identified its central part (anchor domain) as responsible for cell periphery association via insertion into the inner leaflet of the plasma membrane, whereas both N- and C-terminus seem to remain oriented towards the cytoplasm (Aberle et al., 2002; Frischmuth et al., 2004; Zhang et al., 2002, Fig. 1A). Upon expression in yeast, MP targeted to the plasma membrane, even if fused to a nuclear localization signal, a circumstance that precluded application of the complete protein in a GAL4-based two-hybrid system (Frischmuth et al., 2004). Consequently, only the N-terminal domain (amino acid positions 1–116) was used as a bait to identify interacting plant factors in a cDNA library of the non-host plant *Arabidopsis*. Three different classes of MP-interacting factors comprising 129 independent clones were confirmed by repeatedly monitoring histidine prototrophy and β-galactosidase activity (Fig. 1B, data not shown). Among these, a partial cDNA of 0.7 kb was identified that encodes the C-terminal part of a nuclear-encoded plastid-targeted HSC70 (At4g24280, cpHSC70-1 amino acid positions 469–718). Fig. 1B summarizes the results for this MP-interacting partner. Neither MP_{1-116} nor cpHSC70-$1_{469-718}$ two-hybrid constructs activated the reporter genes in combination with the controls, supporting a specific binding between MP and cpHSC70-1.

Protein interactions of cpHSC70-1 and AbMV MP in planta

In order to examine the association of AbMV MP with cpHSC70-1, the individual homooligomerization potential as well as the subcellular localization of interactions in living plant cells, BiFC was applied

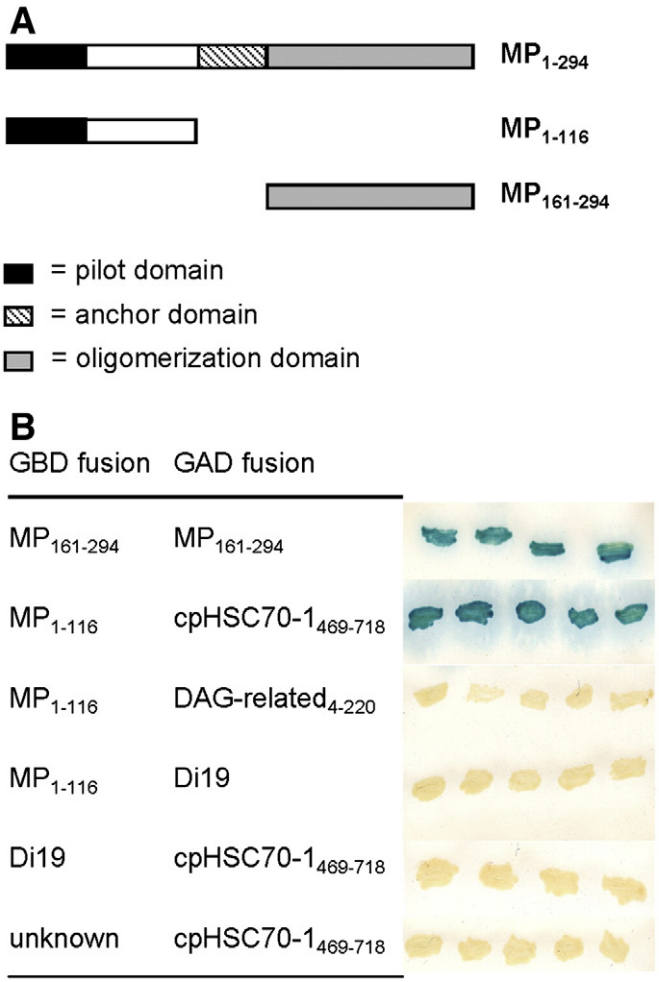


Fig. 1. The C-terminal part of cpHSC70-1 interacts in the yeast two-hybrid system with the N-terminal domain of AbMV MP. (A) Schematic representation of full-length MP (MP_{1-294}) and the used MP truncations (MP_{1-116} and $MP_{161-294}$). Functions assigned to MP regions are indicated (Frischmuth et al., 2004; Zhang et al., 2002). (B) Qualitative β-galactosidase enzyme filter lift assay on the interaction between GBD:MP_{1-116} and GAD:cpHSC70-$1_{469-718}$. As negative controls GAD or GBD fusions of a chloroplast developmental protein (DAG-related, At2g33430), a drought-induced protein (Di19, At1g56280), and an unknown, putatively chloroplast-localized protein (At1g78110) were tested in parallel. The verified MP homooligomerization via the C-terminal domain (Frischmuth et al., 2004) served as positive control.

(Schütze et al., 2009; Walter et al., 2004). Fusion proteins of full-length MP or cpHSC70-1 with either the N- or the C-terminal half of yellow fluorescent protein (YFP^N or YFP^C, respectively) were expressed in pairwise combinations using agro-infiltration of *N. benthamiana* leaves. As controls, single BiFC constructs of cpHSC70-1 or MP were infiltrated together with the complementing "empty" vectors expressing free YFP^N or YFP^C, respectively. In none of these control combinations did any reconstitution of YFP fluorescence between days 2 and 4 post infiltration occurred (data not shown). For maximization of the YFP^N- and YFP^C-tagged protein content, the silencing suppressor p19 of *Cymbidium* ringspot virus was co-expressed (Kleinow et al., 2009b; Lakatos et al., 2004). Protein levels were analyzed by western blots (data not shown). To obtain comparative BiFC samples of healthy and locally AbMV-infected leaf tissues, experiments were carried out either without or with simultaneous agro-infiltration of infectious AbMV DNA A and DNA B constructs.

When MP:YFP^N and MP:YFP^C were co-expressed, a fluorescence signal, either with an even appearance or in small-sized spots at the cell periphery, was observed in approximately 5% of cells within the infiltrated area (Fig. 2A). In contrast, if MP:YFP^N and MP:YFP^C

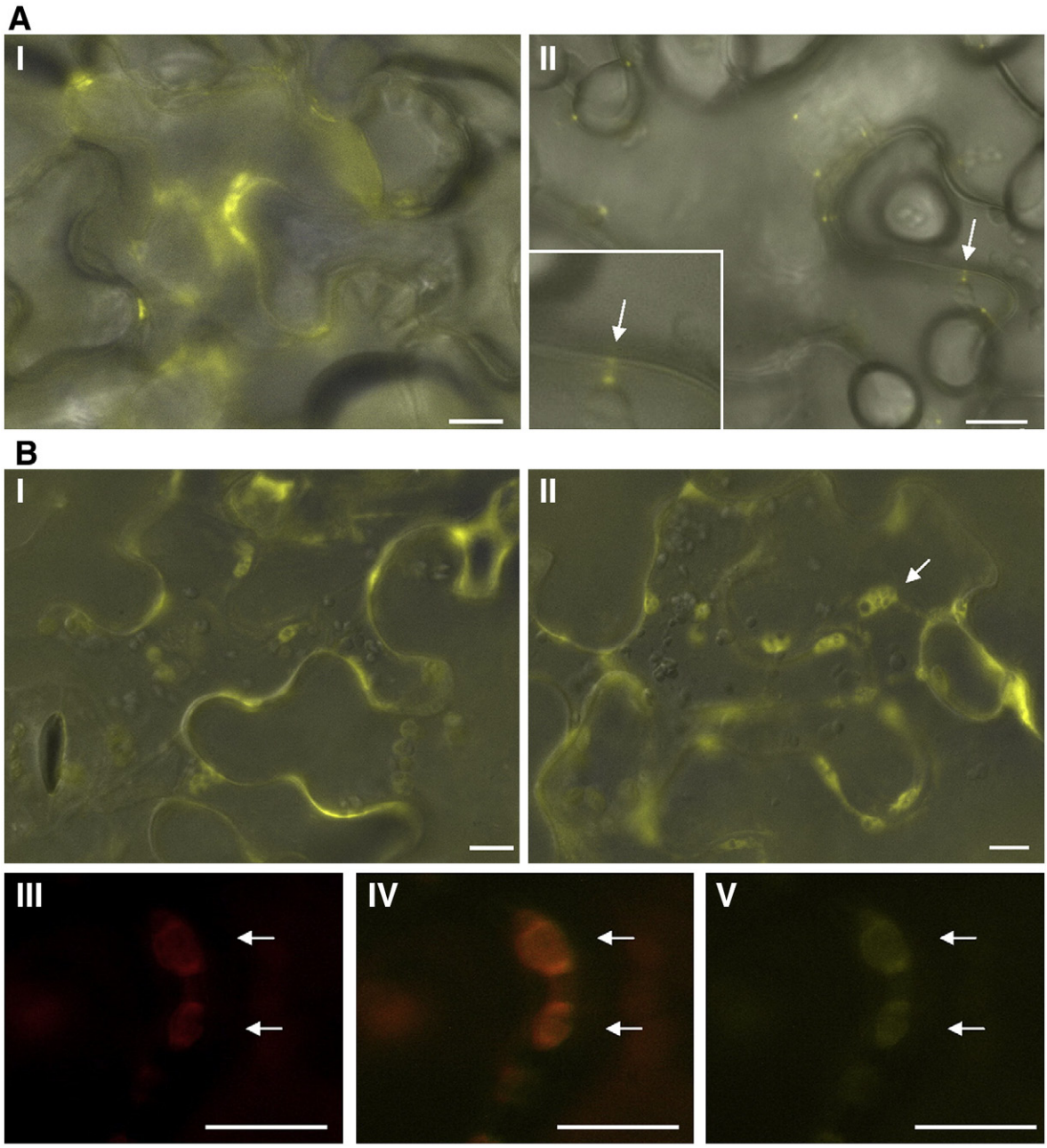


Fig. 2. Visualization of AbMV MP homooligomerization in healthy or AbMV-infected *N. benthamiana* by BiFC. (A) Merged YFP fluorescence and bright field images (I and II) of leaf cells 3 days after infiltration with *A. tumefaciens* harboring expression constructs for full-length MP fused to YFP N-, or C-terminal half, respectively (YFP^N or YFP^C). Inset in (II): a close-up of the right spot (arrow). (B) Co-infiltration as in (A), but with infectious AbMV DNA A and DNA B constructs added. Panels (I) and (II) show merged YFP fluorescence and bright field images, and panels (III)–(V) close-ups of chloroplasts inspected either for chlorophyll autofluorescence (III), or YFP emission (V) at 4 dpi. (IV) represents superposition of YFP and chlorophyll autofluorescence images. Arrows indicate a morphologically altered, vesiculated plastid (II), or plastids exhibiting autofluorescence and YFP emission (III–V). Scale bars, 10 µm.

constructs were co-infiltrated together with infectious DNA A and DNA B, the amount of BiFC-positive cells was significantly increased (up to 40%) in the locally AbMV-infected tissue (Fig. 2B). As in the healthy leaf, BiFC signals were present at the cell periphery (Fig. 2B, I and II), but additional signals occurred associated with plastids as indicated by superposition of YFP and chlorophyll autofluorescence (Fig. 2B arrows, III–V). In summary, BiFC confirmed a homooligomerization of AbMV MP *in planta*, which has been detected in yeast before (Frischmuth et al., 2004). The observed vesiculated entities of BiFC-positive plastids during local AbMV infection (Fig. 2B, II, arrow) resembled those of plastids from systemically AbMV-infected plants investigated previously by electron microscopy (Jeske and Werz, 1978; Schuchalter-Eicke and Jeske, 1983). On the fluorescence microscopy level, nevertheless, it was hard to decide whether the MP oligomer-specific signal stemmed from the inner or the outer side of the plastids, especially because invaginations of cytoplasm into the plastids may occur (Jeske and Werz, 1978).

When MP:YFP^C was combined with cpHSC70-1:YFP^N, peripheral YFP signals were detectable in around 5% of the cells (Fig. 3A). However, a local AbMV infection, again, enhanced significantly the number of BiFC-positive cells (up to 45%). Specific fluorescence appeared clearly associated with the cell periphery (Fig. 3B, I and II) as well as with plastids (Fig. 3B, III–V, arrows). Merging the chlorophyll autofluorescence with the YFP signal confirmed the localization at plastids (Fig. 3B,

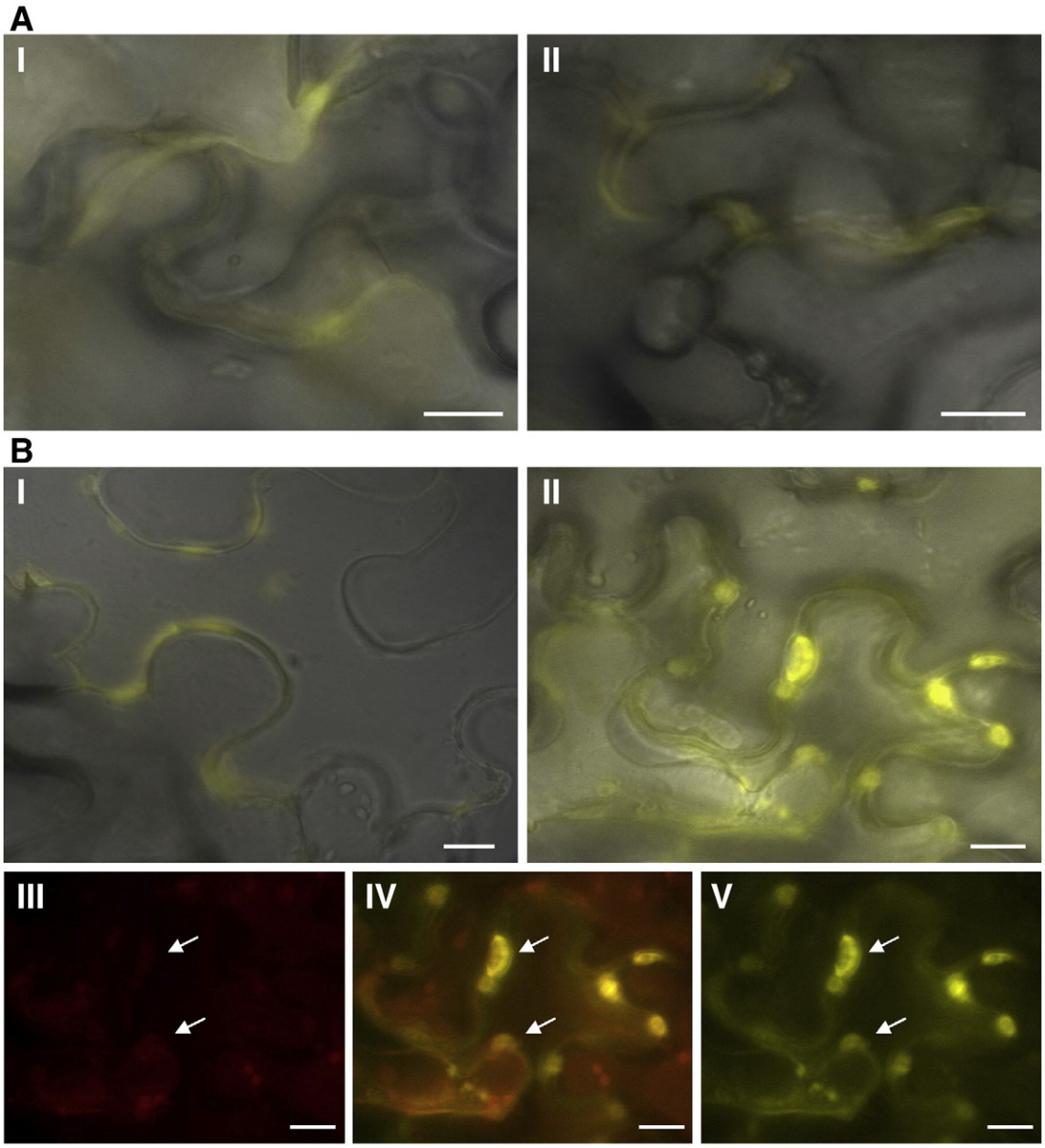


Fig. 3. Interaction between AbMV MP and cpHSC70-1 full-length proteins visualized via BiFC in healthy or AbMV-infected *N. benthamiana*. (A) *N. benthamiana* leaves infiltrated with *Agrobacterium* suspension harboring cpHSC70-1:YFP^N or MP:YFP^C expression constructs. Merged YFP fluorescence and bright field images (I and II) of cells at 4 dpi. (B) Co-infiltration as in (A), but with infectious AbMV DNA A and DNA B constructs added. Panels (I) and (II) show merged YFP fluorescence and bright field images, panel (III)–(V) close-ups of (II) inspected either for chlorophyll autofluorescence (III), or YFP emission (V) at 4 dpi. In (IV) a superposition of images (III) and (V) is displayed. Plastids exhibiting autofluorescence and YFP emission are marked by arrows. Scale bars, 10 µm.

III–V, arrows). Hence, the obtained BiFC results provided evidence for a complex formation between cpHSC70-1 and AbMV MP *in planta*.

Previous reports have localized cpHSC70-1 in the chloroplast stroma (Peltier et al., 2006, 2002; Su and Li, 2008; Sung et al., 2001). Accordingly, in non-infected cells a YFP-label throughout chloroplasts with small bright fluorescent spots has been detected after co-expression of cpHSC70-1:YFP^N and cpHSC70-1:YFP^C, indicating a homooligomerization of this chaperone inside plastids (Fig. 4A). From some plastids, small BiFC-positive filaments arose and extended to the cell periphery, but these did not show chlorophyll autofluorescence (Fig. 4A, arrows). cpHSC70-1 homooligomer-specific fluorescence also appeared close to the cellular margin, either in spots (Fig. 4A, II and V, arrows) or more homogenously distributed (Fig. 4A, II and V). In AbMV-infected plant cells, the subcellular distribution of cpHSC70-1–cpHSC70-1 interaction was altered. The plastid-localized YFP-fluorescent spots appeared as strings of pearls interconnecting different chloroplasts in up to 30% of the BiFC-positive cells at 4 dpi (Fig. 4B, I and II). Some of those strings extended to the cellular margin (Fig. 4B, III and VI, arrows). Also in these structures, no chlorophyll autofluorescence was detectable (Fig. 4B, IV–VI). An equally vesiculated appearance of YFP-labeled plastids was observed as in the MP–MP BiFC studies described above (compare Fig. 4B, III, arrows, to Fig. 2B, II, arrow). Shorter filaments between plastids and the cell periphery, similar to those found in non-infected cells, were detected in a few cases as well (Fig. 4B, III, arrows).

Effects of NbcpHSC70 silencing on AbMV infection

To explore a potential biological relevance of the interaction between cpHSC70-1 and AbMV MP for viral spread or symptom

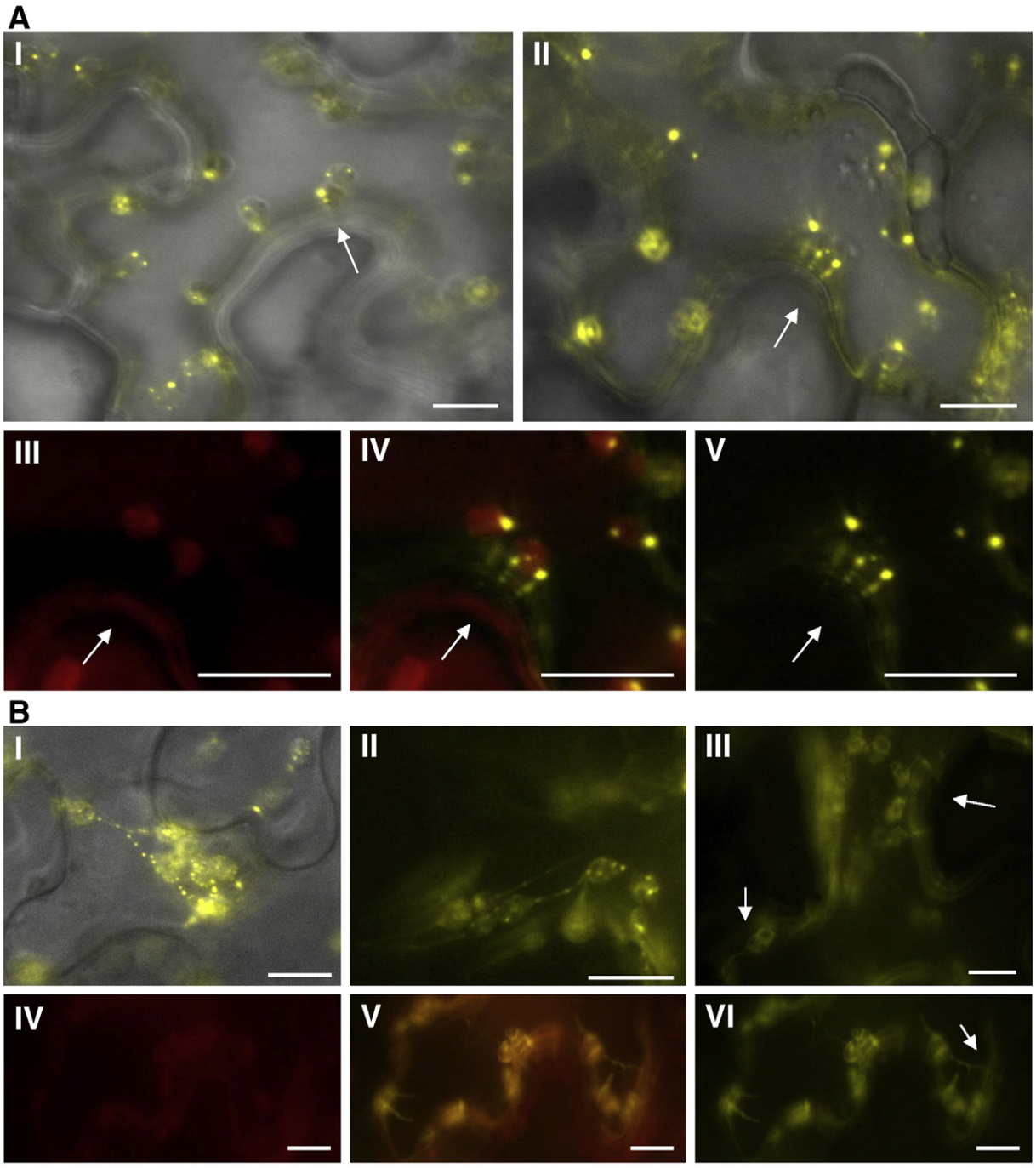
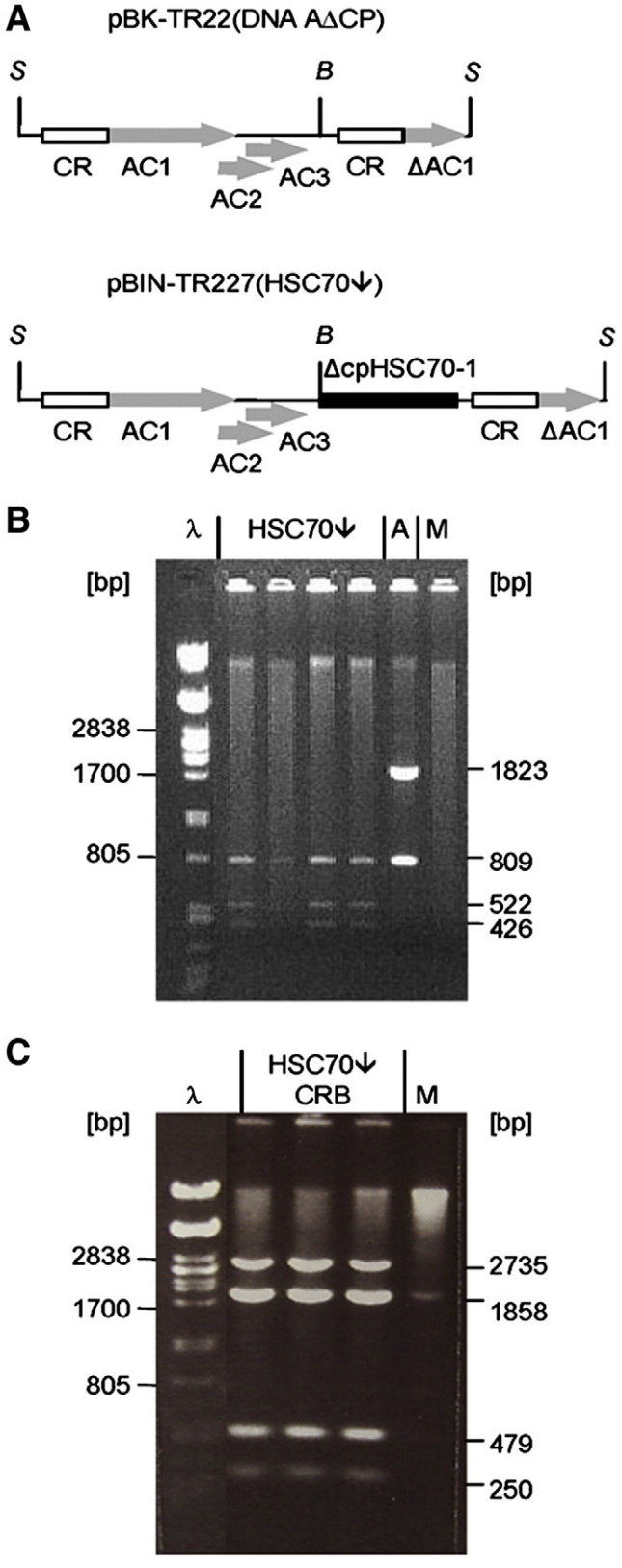


Fig. 4. Visualization of cpHSC-1 homooligomerization in *N. benthamiana* by BiFC in the absence or presence of AbMV infection. (A) Superposition of YFP emission and bright field images (I and II) of leaf cells agro-infiltrated with expression constructs of full-length cpHSC70-1, fused to YFP^N or YFP^C, at 3 dpi. The lower row depicts a close-up of (II): image (III) shows chlorophyll autofluorescence, image (V) YFP fluorescence, and image (IV) the merged pictures (III) and (V). Small YFP-fluorescent filaments connecting plastids and cell periphery are labeled by arrows. (B) Additionally to the combination used in (A), infectious AbMV DNA A and B constructs were agro-infiltrated and leaves analyzed at 4 dpi. Merged epifluorescence and bright field images (I), or YFP images (II, III and VI) of cpHSC70-1 oligomers localized in strings of pearls interconnecting different plastids (I and II), in filaments extending to the periphery (arrows in III and VI) and at the cell periphery (III and VI). In the lower row, image (IV) shows chlorophyll autofluorescence, image (VI) YFP fluorescence, and the center a merged image (V). Scale bars, 10 µm.

development, we applied a reverse genetic approach. The 0.7 kb cpHSC70-1 cDNA fragment of *Arabidopsis* was subcloned from the two-hybrid vector into an AbMV DNA A-based virus-induced gene silencing (VIGS) vector (Fig. 5A, Krenz, 2007). Based on the nucleotide sequence identity, a specific silencing of the homologous cpHSC70 gene (NbcpHSC70, AB181295) in the host plant *N. benthamiana* was expected by the recombinant NbcpHSC70 silencing replicon [pBIN-TR227(HSC70↓)]. The VIGS construct was first tested for its virus-like infectivity by delivery into *N. benthamiana* transgenic for a dimeric copy of AbMV DNA B and thereby capable for release of a stable DNA B replicon in the presence of a DNA A, resulting in a systemic infection ("DNA B plants", Krenz, 2007; Wege and Pohl, 2007, Fig. 5B). Stems of DNA B plants were inoculated with agrobacteria either harboring the NbcpHSC70 silencing construct, an infectious AbMV DNA A clone, or an AbMV DNA A-based VIGS vector that silences phytoene desaturase (PDS) expression [pBIN-TR229(PDS↓)] for comparison, respectively (Krenz, 2007). To assay infectivity in a pseudorecombination, pBIN-TR227(HSC70↓) was co-inoculated with an agro-infectious clone of

the related Sida golden mosaic Costa Rica virus (SiGMCRV) DNA B in stems of wild-type *N. benthamiana* (Fig. 5C). Plants treated with water served as negative control ("mock"). Total nucleic acids extracted at 21 days post infection (dpi) from newly emerged sink leaves were analyzed for presence of circular viral DNAs by ϕ29 polymerase-based rolling circle amplification (RCA) and restriction fragment length polymorphism (RFLP, Haible et al., 2006). Following endonuclease digestion samples yielded the expected band patterns for AbMV DNA A, or the respective PDS or NbcpHSC70 silencing replicons demonstrating the systemic infectivity (Figs. 5B and C, Krenz, 2007). Samples from mock-treated control plants generated no RCA product. RCA–RFLP and tissue print blots probed with AbMV DNA A confirmed systemic infection by the cpHSC70 silencing replicon with usually 100% infection rate per independent experiment (example in Figs. 5B and C, data not shown).



At 21 dpi, AbMV DNA A-infected DNA B plants revealed the typical symptoms of an AbMV infection such as yellow-green leaf mosaic and lamina curvatures (Fig. 6A). Plants inoculated with the PDS silencing construct showed the expected development of large bleached leaf sectors (Fig. 6A, Krenz, 2007) indicating effective silencing by the AbMV DNA A-based VIGS vector. The growth habit of pBIN-TR227 (HSC70↓)-inoculated DNA B plants was indistinguishable from mock-treated ones, but single white spots emerged adjacent to major veins of newly developing leaves (Fig. 6A, arrows). Microscopy of these white areas revealed that DAPI-stained nuclei exhibited fluorescence as in green tissues (data not shown), whereas chlorophyll autofluorescence was considerably decreased (Figs. 7A and B). Remarkably, if the NbcpHSC70 silencing replicon was combined with SiGMCRV DNA B the chlorotic silencing phenotype was not restricted to spots adjacent to major veins, instead whole leaves and stems were bleached comparable to PDS silencing (Fig. 6). Thus the AbMV DNA A-based cpHSC70-1 VIGS construct possessed the capability to comprehensively silence the cpHSC70 expression in *N. benthamiana* if combined with a non-AbMV DNA B. To determine whether in the white leaf tissue indeed the NbcpHSC70 mRNA level was strongly reduced, a semi-quantitative RT-PCR was performed. The NbcpHSC70 transcript was detected in mock-, pBIN-TR229(PDS↓)-, and AbMV DNA A-infected plants, but was absent in pBIN-TR227(HSC70↓)-inoculated ones (Fig. 7C). Complementary, pBIN-TR227(HSC70↓)-infected plants displayed small interfering RNAs (siRNA) specific for the NbcpHSC70 mRNA in northern blots, which were absent from the controls (Fig. 7D). The results indicate a silencing of NbcpHSC70, which had most likely interfered with chloroplast stability within plant cells (Figs. 6 and 7A and B).

For further investigation why NbcpHSC70 silencing was restricted to small-sized spots in combination with AbMV DNA B, DNA B plants either mock-treated or agro-inoculated with pBIN-TR227(HSC70↓), -TR229(PDS↓), -TR22(DNA AΔCP), or AbMV DNA A were analyzed at 60 dpi. Nearly complete photo-bleaching of whole leaves and stems was observed in the plants infected systemically with the PDS silencing replicon (Krenz, 2007). As before, the pBIN-TR227(HSC70↓)-inoculated plants showed only minute bleached spots which never

Fig. 5. Infection potential of the recombinant virus construct. (A) Schematic representation of AbMV DNA A-based replicons. pBK-TR22 contains a partial dimer of AbMV DNA A with the coat protein gene deleted, and an additional BamHI cloning site (B) flanked by SalI restriction sites (S).To generate a NbcpHSC70 silencing replicon (HSC70↓) a 715 bp fragment of *A. thaliana* cpHSC70-1 cDNA was inserted into the BamHI site of pBK-TR22 and the resulting cassette was transferred into pBINplus to yield the agro-infectious VIGS vector pBIN-TR227(HSC70↓). Upon delivery into plant cells a stable replicon 103 bp larger than AbMV DNA A will be released. 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. (B) Viral DNAs were extracted at 21 dpi from *N. benthamiana* (DNA B plants) either mock-treated (M) or systemically infected with AbMV DNA A (A), or pBIN-TR227(HSC70↓), respectively, and analyzed by RCA–RFLP using EcoRI. Samples were separated on a 1% agarose gel (molecular mass marker: PstI-digested Lambda DNA [λ]). Expected restriction fragments are indicated (AbMV DNA A: 1823 and 809 bp; NbcpHSC70 silencing replicon: 809, 522, 426 and 117 bp; 117 bp not visible in Panel B). AbMV DNA B is unaffected by EcoRI. (C) Viral DNAs extracted from plants either mock-treated (M), or infected with SiGMCRV DNA B (CRB) and pBIN-TR227 (HSC70↓), respectively, were analyzed for construct integrity by RCA–RFLP using BamHI at 20 dpi. Samples were separated on a 1% agarose gel. Sizes of molecular mass marker (λ, PstI-digested Lambda DNA) and expected restriction fragments (NbcpHSC70 silencing replicon: 2735 bp and SiGMCRV DNA B: 1858, 479 and 250 bp) are indicated.

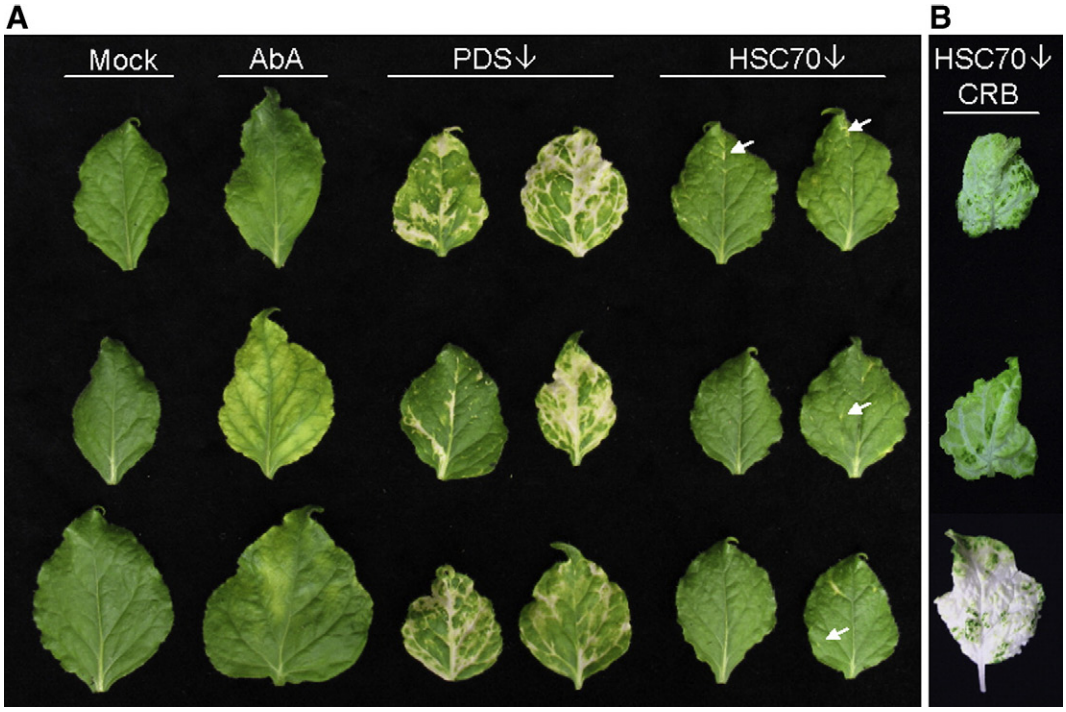


Fig. 6. Phenotypic differences between NbcpHSC70 and PDS silencing, and NbcpHSC70 silencing phenotype in combination with SiGMCRV DNA B. (A) Leaf phenotypes from *N. benthamiana* (DNA B plants) 60 days after mock-infection or inoculation with binary vectors carrying a cpHSC70 silencing replicon (HSC70↓, pBIN-TR227), a PDS silencing replicon (PDS↓, pBIN-TR229; Krenz, 2007) or AbMV DNA A (AbA). White arrows indicate single white spots next to major veins. (B) Systemically infected leaves of wild-type *N. benthamiana* co-inoculated with pBIN-TR227 (HSC70↓) and SiGMCRV DNA B (CRB) at 60 (upper and middle) or 20 (lower) dpi.

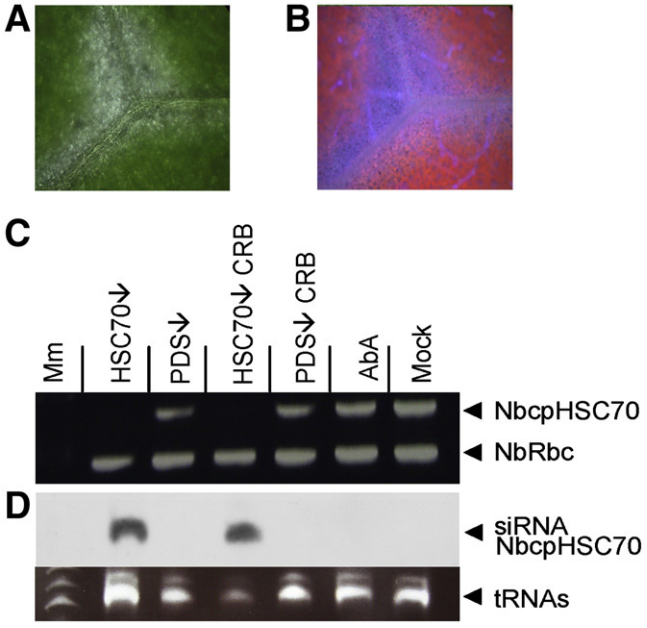


Fig. 7. Silencing effects of the NbcpHSC70 replicon in *N. benthamiana*. (A) Chlorotic leaf area surrounding minor vein branching from a DNA B plant systemically infected with NbcpHSC70 silencing replicon at 60 dpi (bright field image). (B) Same area under UV light. (C) Total RNAs extracted from *N. benthamiana* (DNA B plants) inoculated with pBIN-TR227 (HSC70↓) pBIN-TR229 (PDS↓), AbMV DNA A (AbA), or mock for control, or wild-type *N. benthamiana* infected with pBIN-TR227 (HSC70↓ CRB) or pBIN-TR229 (PDS↓ CRB) in combination with the related SiGMCRV DNA B were tested by multiplex semi-quantitative RT-PCR for the presence of NbcpHSC70 and RbcS transcripts. Products were separated on a 1% agarose gel (molecular mass marker: 50 bp ladder, New England Biolabs [Mm]). (D) RNA of the same tissue samples as in (C) was tested for the presence of NbcpHSC70-specific siRNA by northern blot analysis. EtBr-stained tRNA bands are shown as loading control.

increased in size, as opposed to the continuous enlargement of chlorotic leaf areas of PDS-silenced plants during the course of the experiment (Fig. 6A). The viral DNA content in systemically infected leaves from four individual plants was monitored by semi-quantitative Southern blot analysis (Fig. 8). Within the white sectors of PDS- and NbcpHSC70-silenced leaves the accumulation of replicative intermediates from DNA A-based silencing replicons as well as DNA B was similar and, moreover, viral double-stranded DNA (dsDNA) levels were comparable to those of wild-type AbMV infection. However, both silencing constructs showed in comparison to the wild-type infection a reduction in the amount of single-stranded DNA (ssDNA), what may either reflect an enhanced instability of unencapsidated ssDNA as discussed (Unseld et al., 2004), or some regulatory effects of CP during the switch from viral dsDNA to ssDNA replication (Stanley and Townsend, 1986). Comparing the chlorotic and green parts within PDS- or NbcpHSC70-silenced leaves, the green tissue revealed either a strongly reduced or an undetectable viral DNA level. Thus, the PDS or NbcpHSC70 silencing replicon content correlated with the appearance of bleached leaf areas. Remarkably, although the viral DNA accumulation was not abolished within white NbcpHSC70-silenced spots, these exhibited a substantial reduction of viral DNA spread.

Discussion

The *Arabidopsis* HSP70 family comprises of 13 genes of which two encode plastid-targeted proteins (Sung et al., 2001). By means of a two-hybrid screen only a single representative (cpHSC70-1) was isolated as AbMV MP-interacting partner. HSP70s possess an N-terminal nucleotide binding domain, a substrate binding domain (SBD), and a variable C-terminal domain (CTD), which is not conserved for family members with different subcellular distributions (Mayer and Bukau, 2005). The truncated cDNA of cpHSC70-1 obtained in the two-hybrid screen comprised two thirds of the SBD and the complete CTD coding region. The amino acid sequence identity of this portion with other *Arabidopsis* HSP70s varies between 30 and 40%,

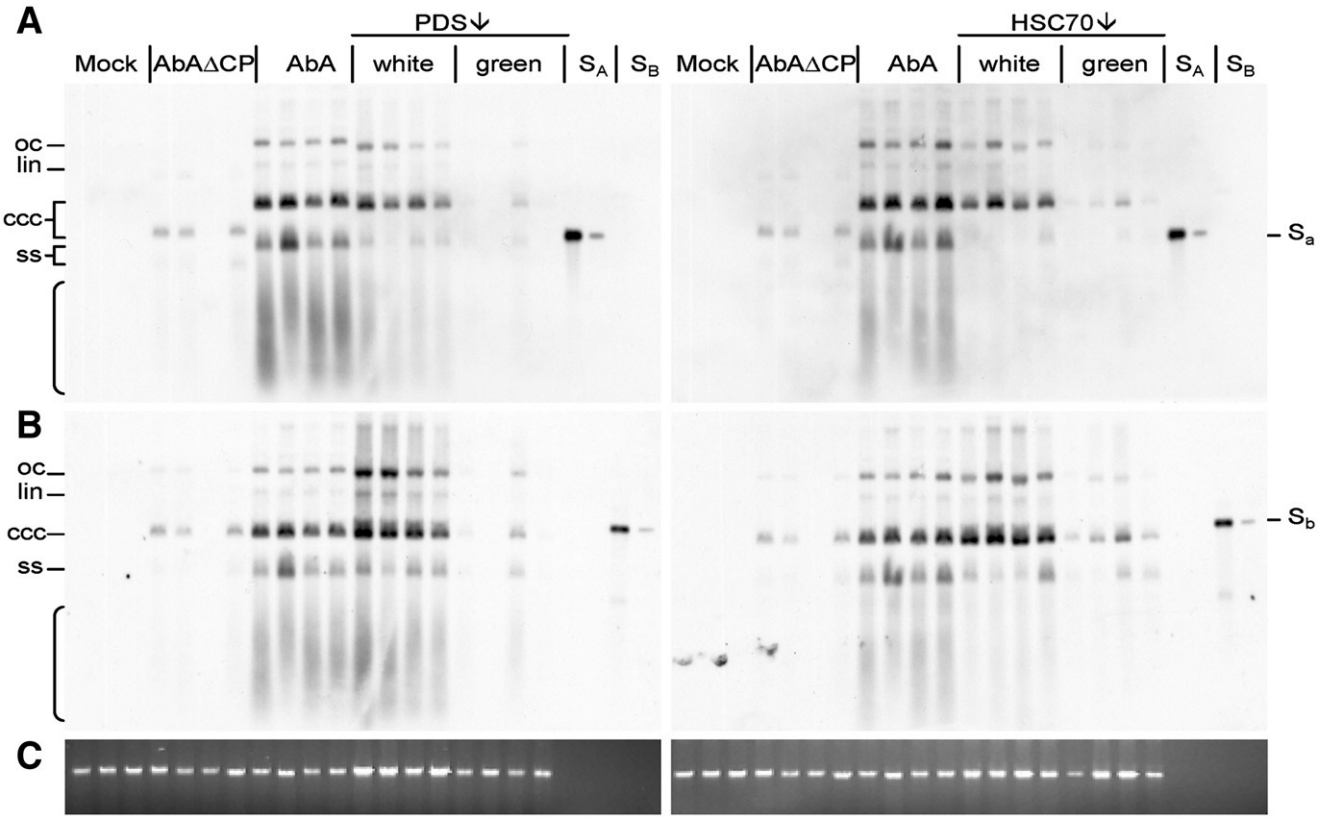


Fig. 8. Viral DNA content of AbMV-infected, PDS- or NbcpHSC70-silenced plants. Total nucleic acid extracted from four *N. benthamiana* (DNA B plants) either mock-treated (Mock) or inoculated with pBIN-TR22 (DNA AΔCP; AbAΔCP), AbMV DNA A (AbA), pBIN-TR229 (PDS↓) or-TR227 (HSC70↓) were separated on a 1% agarose gel in the presence of EtBr. White and green sectors from individual leaves of PDS- or NbcpHSC70-silenced plants were analyzed in parallel. (A) Southern blot hybridized with AbMV DNA A-specific probe. Hybridization standards (left to right 100 and 10 pg of S_A: PCR-amplified DNA A-encoded ORF AC1 or S_B: PCR-amplified DNA B exclusive of common region) and positions of viral DNA forms (oc: open circular, lin: linear, ccc: covalently closed-circular and ss: single-stranded) are indicated. The area corresponding to replication by-products is marked by a bracket. Note that positions of viral DNA forms can vary due to different sizes of the used DNA A derivates. (B) Samples as in (A), but re-probed, after stripping, with the DNA B-specific probe. (C) Gel area representing plant genomic DNA as loading control.

whereas the second plastid-targeted heat shock protein (cpHSC70-2) is more similar (83%). In higher plant chloroplasts, HSP70 isoforms may be located either in the outer envelope (Ko et al., 1992; Schnell et al., 1994), or in the stroma (Marshall and Keegstra, 1992). The best-documented function of HSP70s of the outer envelope is the import of polypeptides into plastids (Soll, 2002; Soll and Schleiff, 2004). In contrast, the function of stroma-targeted HSP70s is less well understood. Potentially, these play a role in protein import into plastids (Su and Li, 2008 and references therein), in photosystem II protection and repair (Schroda et al., 2001, 1999). cpHSC70-1 was confirmed by mass spectrometry to be located in the chloroplast stroma, and import assays indicated its presence therein with truncated transit peptide (Peltier et al., 2006, 2002; Su and Li, 2008; Sung et al., 2001). However, cpHSC70-1 was also found in mitochondria and as a nuclear protein in response to cold stress (Bae et al., 2003; Ito et al., 2006), suggesting localization and possible functions in other cellular compartments, too. The analysis of an *Arabidopsis* T-DNA knock-out (KO) mutant of cpHSC70-1 revealed that its deficiency caused severe developmental defects (Su and Li, 2008). In contrast, a T-DNA mutant of the homologue cpHSC70-2 did not exhibit such dramatic alterations. Double KO mutants turned out to be lethal supporting the assumption that both chaperones have partially overlapping essential functions in plant development.

Several previous studies concluded that plant cytosolic HSP70s might play a role in cell-to-cell movement for virus families other than geminiviruses (Alzhanova et al., 2007; Aoki et al., 2002; Avisar et al., 2008 and references therein; Hofius et al., 2007; Lu et al., 2009; Shimizu et al., 2009; Soellick et al., 2000). Here, we found an AbMV MP-interacting chaperone (cpHSC70-1) which is predicted to be mainly targeted to the chloroplast stroma. Certainly, it is synthesized in the cytoplasm and may interact there with the geminiviral MP during intracellular transit. The relevance of this unexpected interaction was scrutinized *in planta* with both full-length proteins using BiFC, a technique which favors the detection of direct protein–protein interactions (Bhat et al., 2006). However, it is still possible that a third unknown protein that is conserved in yeast and plants may serve as an adapter and the interaction may, thus, be indirect.

The BiFC results showed that homooligomers of cpHSC70-1 accumulated at the expected site in distinct spots closely associated with chloroplasts, but oligomers were also present in small filaments reaching from plastids to the cellular margin and distributed at the cell periphery. In the case of local AbMV infection, cpHSC70-1 complexes appeared as strings of pearls between chloroplasts indicating a viral influence on the subcellular localization. All short filaments observed in non-infected plant cells, and a small fraction of the longer strings of pearls found in infected plant cells extended to the periphery, suggesting a potential function in molecule exchange. Which type of subcellular structure is responsible for the filamental strings of pearls-distribution upon AbMV infection cannot be decided on the light microscopic level and would need further investigation. With respect to form, size and the lack of chlorophyll, the filaments resemble stromules, i.e. stroma-filled plastid tubules (Hanson and Sattarzadeh, 2008; Natesan et al., 2005). The functional role still remains to be determined, but stromules were proposed to participate in chloroplast motility, in facilitating transport of substances into and out of plastids by increasing the surface area, and in exchanging molecules between different organelles (Hanson and Sattarzadeh, 2008; Natesan et al., 2005).

In this study, the BiFC analysis confirmed a homooligomerization of AbMV MP *in planta*, which has been shown before to be mediated by the C-terminal domain in yeast (Frischmuth et al., 2004). The MP oligomers were detected at the cell periphery, either in small spots or more evenly spread, which is in agreement with data obtained previously for the subcellular distribution of either green fluorescent protein (GFP)- or c-Myc-tagged AbMV MP in plant cells (Kleinow et al., 2009b; Zhang et al., 2002, 2001). Depending on the developmental status of the host epidermal cells (*N. benthamiana* and *N. tabacum*) and the time of expression, GFP:MP was found predominantly in punctuate flecks at the cell periphery, but in some cases also uniformly distributed at the periphery and in punctuate bodies around the nucleus (Zhang et al., 2001). In contrast to the results obtained by Zhang et al., here, no BiFC signal was detectable in perinuclear sites, indicating that MP oligomer formation may be restricted to the cell periphery, most likely occurring in the protoplasmic leaflet of plasma membranes (Aberle et al., 2002; Frischmuth et al., 1997, 2004; Kleinow et al., 2009b). Remarkably and unprecedented in the former GFP:MP experiments published, MP oligomerization was also observed at chloroplasts. Whether MP was imported into plastids or just associated with the outer envelope could not be decided on the basis of epifluorescence microscopy. A high non-specific background attachment of antibodies to plastids prevented a more conclusive immunolocalization of AbMV MP by electron microscopy so far (Kleinow et al., 2009b, data not shown).

Moreover, BiFC analysis confirmed a complex formation between cpHSC70-1 and AbMV MP *in planta*. This interaction occurred, similarly to the oligomerization of MP, at the cell periphery and in association with plastids. BiFC signals obtained for all combinations with split YFP:MP fusions tested were significantly enhanced in locally AbMV-infected leaves. This result may reflect stabilization and/or a preferred accumulation of the protein in specific subcellular compartments, promoted by the presence of the cognate viral DNA or other AbMV proteins. In particular the NSP might enable the formation of stable transport complexes.

To further examine the significance of cpHSC70 chaperones for geminiviral movement and symptom development, we used VIGS as a reverse genetic approach. The results confirmed that an *A. thaliana* cpHSC70-1 gene fragment was able to silence the expression of the NbcpHSC70 gene. Similar to PDS silencing (Brigneti et al., 2004; Kumagai et al., 1995; Ruiz et al., 1998; Tao and Zhou, 2004) interfered NbcpHSC70 silencing with chloroplast stability. The white NbcpHSC70-silenced sectors of *N. benthamiana* leaves showed viral DNA accumulation comparable to PDS-silenced or AbMV-infected plants. Nevertheless, in combination with AbMV DNA B the NbcpHSC70 VIGS construct exhibited a spatial restriction to small areas adjacent to veins, suggesting an interference of NbcpHSC70-silencing with viral spread. A complete blocking of the viral movement was not observed, which could have several explanations. First, in spite of silencing of NbcpHSC70 expression minor amounts of protein may be still available, which were sufficient to enable occasional transport. Second, the interaction between cpHSC70 and MP may play a role in a bypass of viral spread and is therefore not an essential prerequisite for it. Third, potential other members of the plastid-targeted HSC70 family could act as redundant factors and rescue the cell-to-cell movement to a certain degree. Currently, only one sequence of a plastid-targeted HSC70 is known for *N. benthamiana*. Whether other members of this subclass exist and if these are affected by pBIN-TR227(HSC70↓) cannot be decided. It is improbable that cytoplasmic HSC70-1 was targeted since it has low sequence similarity to NbcpHSC70. Kanzaki et al. (2003) observed stunting for cytoplasmic HSC70-1-silenced *N. benthamiana*, a phenotype that was absent for NbcpHSC70-silenced plants in this study.

In summary, our experiments yielded several lines of evidence that upon AbMV infection an interaction between the nuclear-encoded and chloroplast-targeted cpHSC70-1 chaperone and AbMV MP occurs and may be functionally relevant: BiFC analysis has confirmed a complex formation between both proteins *in planta*, and AbMV infection has been shown to affect the subcellular distribution of cpHSC70-1 oligomers. VIGS assays provided a first indication for an influence of NbcpHSC70 on AbMV spread.

Materials and methods

Virus strains, plant material and infection

Viral genome components of AbMV (DNA A X15983, DNA B X15984, agro-infectious pBIN19 clones, Frischmuth et al., 1993), DNA B of SiGMCRV (X99551, Frischmuth et al., 1997; Unseld et al., 2000), and pBIN-TR22 and pBIN-TR229 (Krenz, 2007) were used as agro-infectious clones. Wild-type *N. benthamiana* Domin and *N. benthamiana* plants transgenic for a dimeric copy of AbMV DNA B ("DNA B plants", Wege and Pohl, 2007) were grown in an S2 greenhouse with supplementary lighting. Infiltration assays, biolistic or stem inoculation of plants were performed as described (Klinkenberg et al., 1989; Krenz, 2007; Morilla et al., 2004; Zhang et al., 2001).

Cloning procedures

The cpHSC70-1 (At4g24280) ORF without stop codon was PCR-amplified (31 cycles of 10 s at 94 °C, 30 s at 56 °C, and 2 min at 68 °C followed by 7 min at 72 °C) from an *Arabidopsis* cDNA library (Umeda et al., 1998) using the following primers, which add attB sites (underlined) for use in the GATEWAY recombination system (Invitrogen, Carlsbad, CA): 5′-GGGGACAAGTTTGTACAAAAAAG-CAGGCTTCATGGCATCTTCAGCGCCC-3′ and 5′-GGGGACCACTTTGTA-CAAGAAAGCTGGGTCTTGGCTGTCTGTGAAGTCAG-3′. The PCR product was recombined into pDONOR207 according to the manufacturer's protocol (Invitrogen), fully sequenced and transferred into binary BiFC plasmids pSPYCE-35S^{GATEWAY} and pSYPNE-35S^{GATEWAY} (Schütze et al., 2009; Walter et al., 2004). pAS2-1 (Trp^{+}, GAL4 DNA-binding domain [GBD]) and pACT2 (Leu^{+}, GAL4 activation domain [GAD]) two-hybrid constructs used in this study were described previously (Frischmuth et al., 2004; Kleinow, 2000). To create a GATEWAY entry clone of AbMV MP, the full-length coding region without stop codon was amplified by PCR (25 cycles of 10 s at 94 °C, 1 min at 58 °C, and 2 min at 68 °C followed by 5 min at 68 °C) from the agro-infectious DNA B clone (Frischmuth et al., 1993) with the attB site (underlined) adding primer pair 5′-GGGGACAAGTTTGTA-CAAAAAAGCAGGCTTCATGGATTCTCAGTTAGTAAAT-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTCAATGATTTGGCTT-GAG-3′. The product was recombined into pDONOR207, verified by sequencing and transferred to binary BiFC vectors pSPYCE-35S^{GATEWAY} and pSYPNE-35S^{GATEWAY} (Schütze et al., 2009; Walter et al., 2004). VIGS vector pBIN-TR227 for agro-inoculation was generated by releasing the 715 bp C-terminal fragment of cpHSC70-1 by BamHI and BglII restriction from pACT2 and inserting it into BamHI-linearized pBK-TR22 (Krenz, 2007). pBIN-TR227 (Fig. 5) was constructed by excising the AbMV DNA A ΔCP: cpHSC70-1 bitmer cassette from pBK-TR227 by SalI and transferring it into the single SalI site of pBINplus (van Engelen et al., 1995). The resulting binary constructs of cpHSC70-1 and AbMV MP were introduced into *Agrobacterium tumefaciens* GV3101 (pMP90) strain (Koncz et al., 1994) by chemical transformation; construct integrity was confirmed by PCR.

Semi-quantitative Southern blot analysis of viral DNA

Total nucleic acids from systemically infected plant tissues were isolated using a CTAB-based extraction protocol (Kleinow et al., 2009a). Samples of 1 µg total nucleic acid were separated on 1% agarose gels in the presence of ethidium bromide (EtBr), transferred

onto nylon membranes and hybridized with random-primed digoxigenin-labeled probes selective for DNA A (PCR-amplified ORF AC1) and DNA B (PCR-amplified DNA B exclusive of common region) according to Kleinow et al. (2009a).

RCA–RFLP

For RCA of geminiviral DNAs, 10 ng of total nucleic acids isolated as stated above was used per TempliPhi DNA amplification kit reaction and amplification was carried out according to the supplier's protocol (GE Healthcare/Amersham Biosciences, Uppsala, Sweden). RCA products were analyzed for RFLP using the indicated restriction endonucleases according to the manufacturer's recommendation.

RT-PCR and northern blotting of siRNA

Total RNA from 100 mg young leaf material was extracted by TRIZOL reagent according to the supplier's recommendations (Invitrogen). In the case of pBIN-TR227 and pBIN-TR229, RNA was isolated from photo-bleached leaf areas. For RT-PCR, 2 µg of RNA was applied as template for first-strand cDNA synthesis using d(T)18-primers and SuperScript® reverse transcriptase (Invitrogen). PCR amplification (3 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 60 °C, and 1 min 72 °C followed by 5 min at 72 °C) of the 3′end portion of the *N. benthamiana* cpHSC70 (NbcpHSC70, AB181295) cDNA was performed with gene-specific primers: 5′-GTGGAGTCATGACCAAAATTATCCCAAG-3′ and 5′-GAAGTCTGCATCGATAACTTCTCCATC-3′. Ribulose-1,5-bisphosphate carboxylase small subunit mRNA (RbcS; X02353) served as an internal control and was amplified with primer pair: 5′-CCTCTGCAG-CAGTTGCCACC-3′ and 5′-CCTGTGGGTATGCCTTCTTC-3′. The resulting products were analyzed by agarose gel electrophoresis, subcloned into pGEM®-T Easy (Promega Inc., Madison, WI) and verified by sequencing. Northern blotting of siRNA was done as described (Krenz, 2007).

Protein interaction assays using yeast two-hybrid system

A cDNA library prepared from *Arabidopsis* cell suspension culture in the yeast two-hybrid prey vector pACT2 (GAD, Leu^+) was screened for AbMV MP N-terminal domain-interacting host factors using pAS2-1-$BC1_{1-116}$ (GBD, Trp^+) as a bait as described previously (Farras et al., 2001). Bait and prey plasmids were co-transformed into *Saccharomyces cerevisiae* strain Y190 (BD Biosciences/Clontech, Palo Alto, CA) using standard LiCl transformation protocols (Ausubel et al., 1999; Durfee et al., 1993). Transformants were grown for 3–5 days at 30 °C on selective $Trp^-/Leu^-/His^-$SD medium (Rose et al., 1990) containing 50 mM 3-amino-triazole (3-AT). Protein interactions were monitored by LacZ filter lift assays with colonies grown on selective SD medium. A rescued prey plasmid was retransformed into *S. cerevisiae* and interactions assayed repeatedly by mating of Matα strain Y187 (BD Biosciences/Clontech) carrying the pAS2-1 GBD constructs with Mata strain Y190 harboring the pACT2 GAD plasmid according to Matchmaker system manual (BD Biosciences/Clontech).

Bimolecular fluorescence complementation (BiFC) assay

Analysis of protein interactions using BiFC was performed as described by Walter et al (2004) and Schütze et al (2009). Binary plasmids carrying full-length coding regions of AbMV MP or cpHSC70-1 either fused to the N-terminal part of YFP (YFP^N; pSYPNE-$35S^{GATEWAY}$), or to the C-terminal portion of YFP (YFP^C; pSYPCE-$35S^{GATEWAY}$) were introduced into *A. tumefaciens* GV3101 (pMP90) and agro-infiltrated in pairwise combinations along with a 35S promoter-driven expression construct of the silencing suppressor p19 (Lakatos et al., 2004) into sink leaves of three-week-old *N. benthamiana*. Expression of YFP^C and YFP^N fusion proteins was monitored by western blotting using anti-HA and anti-c-Myc antibodies (Schütze et al., 2009; Walter et al., 2004).

Microscopy

Plants were examined for chlorophyll fluorescence using an Axiophot microscope (Carl Zeiss, Göttingen, Germany; filter cube G 365; excitation filter G 365 nm; beam splitter FT 395 nm; emission filter LP 420 nm). For detection of YFP fluorescence, an Axiovert M 200 microscope (Zeiss; YFP: filter cube F46-003 AHF; excitation filter 500/20 nm; beam splitter 515 LP; emission filter 535/30 nm) was used. Chlorophyll autofluorescence was monitored in the Axiovert M 200 microscope by the filter cube 488010-0000-000 (Zeiss; excitation filter 450/90 nm; beam splitter FT 510; emission filter 515/65 nm).

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