Capsid Protein-Mediated Recruitment of Host DnaJ-Like Proteins Is Required for *Potato Virus Y* Infection in Tobacco Plants[∇]

Daniel Hofius,^{1*†} Annette T. Maier,^{1‡} Christof Dietrich,² Isabel Jungkunz,³ Frederik Börnke,^{1,3} Edgar Maiss,⁴ and Uwe Sonnewald^{1,3}

Institut für Pflanzengenetik und Kulturpflanzenforschung, Corrensstrasse 3, 06466 Gatersleben, Germany¹; DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstr. 7b, D-38124 Braunschweig, Germany²; Lehrstuhl für Biochemie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Staudtstr. 5, 91058 Erlangen, Germany³; and Institute of Plant Diseases and Plant Protection, University of Hanover, Herrenhauser Str. 2, 30419 Hannover, Germany⁴

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The capsid protein (CP) of potyviruses is required for various steps during plant infection, such as virion assembly, cell-to-cell movement, and long-distance transport. This suggests a series of compatible interactions with putative host factors which, however, are largely unknown. By using the yeast two-hybrid system the CP from *Potato virus Y* (PVY) was found to interact with a novel subset of DnaJ-like proteins from tobacco, designated NtCPIPs. Mutational analysis identified the CP core region, previously shown to be essential for virion formation and plasmodesmal trafficking, as the interacting domain. The ability of NtCPIP1 and NtCPIP2a to associate with PVY CP could be confirmed *in vitro* and was additionally verified in planta by bimolecular fluorescence complementation. The biological significance of the interaction was assayed by PVY infection of agroinfiltrated leaves and transgenic tobacco plants that expressed either full-length or J-domain-deficient variants of NtCPIPs. Transient expression of truncated dominant-interfering NtCPIP2a but not of the functional protein resulted in strongly reduced accumulation of PVY in the inoculated leaf. Consistently, stable overexpression of J-domain-deficient variants of NtCPIP1 and NtCPIP2a dramatically increased the virus resistance of various transgenic lines, indicating a critical role of functional NtCPIPs during PVY infection. The negative effect of impaired NtCPIP function on viral pathogenicity seemed to be the consequence of delayed cell-to-cell movement, as visualized by microprojectile bombardment with green fluorescent protein-tagged PVY. Therefore, we propose that NtCPIPs act as important susceptibility factors during PVY infection, possibly by recruiting heat shock protein 70 chaperones for viral assembly and/or cellular spread.

Systemic invasion of plants by viruses depends on compatible interactions between host and virus-encoded factors to facilitate genome replication, cell-to-cell movement via plasmodesmata (PD) and long-distance transport through the vascular tissue (14, 49, 53, 64). For cell-to-cell spread, most viruses possess distinct movement proteins (MPs) that permit intra- and intercellular trafficking of infectious nucleic acids by utilizing components of the existing host cellular transport machinery such as the cytoskeletal network and endomembrane system (7, 8, 36, 45, 47, 48, 54, 68, 74). Among the various virus families, different mechanisms of MP action have evolved (14, 46, 48). In many cases, a single dedicated MP binds directly to the viral genome and modifies the size exclusion limit (SEL) of PD to facilitate trafficking of MP-nucleic acid complexes. Alternatively, some virus families contain a set of different MPs that are proposed to function coordinately in the cell-to-cell transfer of viruses, whereas MPs of other virus groups form PD-associated tubules through which entire virions can move.

Common among many plant viruses is the requirement of the capsid protein (CP) for efficient long-distance transport, which is most likely related to its capacity to form virus particles (52). Consistently, if cell-to-cell transport of the virus genome occurs in an encapsidated form, the CP has often been shown to be essential or to exhibit complementary functions to those of MPs in cell-to-cell movement (12, 64).

Potyviruses comprise the largest genus of plant viruses infecting a broad range of dicot and monocot crops. Their single-stranded positive-sense RNA genome encodes a large polyprotein that is subsequently cleaved by virus-encoded proteinases into nine or more functional polypeptides (67). In contrast to most other virus groups, potyviruses do not encode a dedicated MP, but movement function has been allocated to several proteins with additional roles in the viral infection cycle including the CP (59), the viral genome-linked protein VPg (27, 62), the helper component protease and silencing suppressor HC-Pro (59), and the cylindrical inclusion protein (13). The multifunctional CP is required for both cell-to-cell and long-distance movement, yet not for virus replication, as demonstrated by genetic analyses using an infectious clone of *Tobacco etch virus* (TEV) (23, 24). The CP is a three-domain protein with variable N- and C-terminal domains exposed on the virion surface and a core region that binds RNA. Mutations in the core region of TEV CP revealed an essential role for virus assembly and cell-to-cell movement, suggesting that intercellular transport involves virions. In contrast, the N- and C-terminal regions are dispensable for assembly but are required

* Corresponding author. Mailing address: Copenhagen Biocenter, University of Copenhagen, Ole Maaloes Vej 5, DK-2200 Copenhagen N, Denmark. Phone: 45-35322103. Fax: 45-35322128. E-mail: hofius@my.molbio.ku.dk.

† Present address: Department of Molecular Biology, Copenhagen Biocenter, University of Copenhagen, Ole Maaloes Vej 5, DK-2200 Copenhagen K, Denmark.

‡ Present address: Max-Planck Institut für Entwicklungsbiologie, Spemannstr. 35-39, D-72076 Tübingen, Germany.

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for efficient long-distance transport (23, 24). A distinct MP-like function for potyvirus CPs in cell-to-cell transport has been proposed from microinjection studies with recombinant CPs from *Bean common mosaic necrosis virus* and *Lettuce mosaic virus* demonstrating that CPs are able to modify plasmodesmal SEL and to mediate their own trafficking, as well as the transport of viral RNA from cell to cell (59).

The nature of host factors involved in the various steps of potyvirus infection, in particular during intra- and intercellular trafficking of viruses, is largely unknown (57). Only lately, the eukaryotic translation initiation factor eIF4E, previously implicated mainly in genome replication, and a cysteine-rich plant protein of unknown function have been identified as susceptibility factors supporting potyvirus movement through interaction with the virus genome-linked protein VPg (27, 33). Similarly, a limited number of host proteins have been demonstrated to interact with MPs of other virus groups (7, 35, 48, 49, 53, 54, 64). For instance, cell wall-associated pectin methylesterase and the endoplasmic reticulum-localized proteins calreticulin and NtCAPP1 involved in the plasmodesmal transport pathway have been isolated as host proteins contributing to cell-to-cell transport of *Tobacco mosaic virus* (TMV) through interaction with the MP (15, 16, 26, 47). NbNACa1, homologous to the alpha chain of nascent-polypeptide-associated complex, has recently been demonstrated to interact with *Brome mosaic virus* (BMV) MP and to be essential for BMV movement (43). Furthermore, a mammalian rab acceptor-like protein, a homeodomain protein, and an ankyrin repeat-containing protein have been reported to associate with MPs of *Cauliflower mosaic virus* (CaMV), *Tomato bushy stunt virus*, and *Potato virus X*, respectively (19, 31, 41). However, the biological role for most of these unrelated host proteins during virus spread has yet to be elucidated. In addition, screening of a yeast two-hybrid library with the *Tomato spotted wilt tospovirus* (TSWV) MP (NSm) led to the isolation of a myosin/kinesin-like protein, as well as DnaJ-like (HSP40) proteins from different plant species, suggesting the involvement of the cytoskeleton and the recruitment of HSP70-related chaperones in TSWV movement (66, 70). A direct role of molecular chaperones in virus movement was corroborated by functional analysis of a HSP70 homolog encoded by closteroviruses that was demonstrated to associate with PD and to exhibit distinct functions in virion assembly and cell-to-cell movement (2, 51, 55). Induction of host HSP70 gene expression after infection with a range of different plant viruses (4, 29, 73) and demonstration of PD trafficking of HSP70 class proteins from the phloem (3) further suggested that viruses unrelated to closteroviruses may well exploit host cellular HSP70 for virion construction and PD trafficking directly or indirectly through interaction with J-domain proteins (7).

In order to gain more insight into the function of the CP during potyvirus pathogenesis, we used the yeast two-hybrid system to isolate host proteins capable of interacting with the CP from *Potato virus Y* (PVY). We identified a novel subset of DnaJ-like proteins from *N. tabacum*, designated capsid protein interacting proteins (NtCPIPs), that specifically bind to PVY CP in yeast and in vitro. The interaction could be confirmed in planta by bimolecular fluorescence complementation (BiFC) analysis, and its biological relevance was verified by infection of tobacco plants ectopically expressing dominant-negative mu-

nants of NtCPIPs. As a consequence of impaired NtCPIP function, transgenic plants showed a strong increase in virus resistance, probably due to reduced cell-to-cell spread of PVY. This suggested the requirement and CP-mediated recruitment of host HSP70-related chaperones for potyviral pathogenesis.

MATERIALS AND METHODS

Plant material and growth conditions. Tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN) were grown in tissue culture under a 16-h light/8-h dark regime (irradiance 150 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) at 50% humidity on Murashige Skoog medium (Sigma) containing 2% (wt/vol) sucrose. *N. tabacum* and *N. benthamiana* plants were kept in soil under greenhouse conditions with 16 h of supplementary light (200 to 300 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) and 8 h of darkness. The relative humidity varied between 60 and 70%, and temperatures were adjusted to 22 and 18°C during the light and dark periods, respectively. The transgenic control line ME-4, expressing the β -glucuronidase (GUS) reporter gene under control of the source-leaf specific *FBPase* promoter was described previously (28). Transgenic tobacco plants constitutively expressing the HPV L1 coat protein were introduced before (6).

PVY CP cDNA isolation. PVY CP encoding cDNA was amplified by reverse transcription-PCR from total RNA extracted from PVY^N (N-strain)-infected tobacco leaf tissue (37). Specific oligonucleotides (5'-ATGAATTCGCAATGACACAATTGATGC-3' and 5'-ATGTCGACCATGTTCTTGACTCCAAGTAG-3') were deduced from a published PVY (N strain) sequence (58) (GenBank accession no. D00441) and designed to introduce EcoRI and SalI restriction sites for further cloning steps. The PCR fragment was inserted into the pGEM/T vector (Promega, Inc., Madison, WI), and the sequence was determined (GenBank accession no. AY319647).

Yeast two-hybrid assays. Yeast two-hybrid screening was performed by using a GAL4-based system (30) and the yeast strain Y190 (34). An oriented activation domain (AD)-tagged cDNA library (10^7 PFU) was constructed from *N. tabacum* source leaf material by using the HybriZAP kit with the pAD-GAL4 vector (Stratagene, La Jolla, CA) and converted to a yeast plasmid library by in vivo excision according to the manufacturer's instructions. The EcoRI/SalI-flanked PVY CP cDNA was cloned into the GAL4-binding domain (BD) vector pGBT9 (BD Biosciences/Clontech, Palo Alto, CA) to produce BD-PVY CP, which was used as bait. The library was transformed into the yeast reporter strain containing BD-PVY CP by the PEG/LiAC/ssDNA method described previously (63). Transformants were cultured for 7 to 10 days at 30°C on synthetic dropout (SD) medium lacking tryptophan, leucine, and histidine ($\text{Trp}^-/\text{Leu}^-/\text{His}^-$) and supplemented with 25 mM 3-aminotriazole (Sigma, St. Louis, MO). Growing colonies were tested for *lacZ* activity by a X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) filter staining assay (5), followed by the preparation of plasmids from positive clones. Unrelated sequences, those of the murine protein p53 (42) and the yeast proteins SNF1 and SNF4 (30), were used as negative and positive interaction controls, respectively. Direct interaction of two proteins was investigated by cotransformation of the respective plasmids in the yeast strain Y190, followed by selection for transformants on SD $\text{Trp}^-/\text{Leu}^-/\text{His}^-$ for 3 to 4 days and subsequent transfer to SD $\text{Trp}^-/\text{Leu}^-/\text{His}^-$ for growth selection and *lacZ* activity testing of interacting clones.

For analysis of the interaction ability of NtCPIP2a and NtCPIP2b, cDNA fragments were amplified from the identified cDNA library clones that lacked in analogy to the isolated NtCPIP1 two-hybrid clones the coding region for amino acids (aa) 1 to 11. PCR products were subcloned into the pCR-Blunt vector (Invitrogen, Carlsbad, CA) and inserted as EcoRI-SalI fragments into the pAD-GAL4 AD vector. Plasmids containing BD-fused TSWV NSm and interacting AD-tagged NtDnaJ_M541 were as described previously (66) and were kindly provided by J.-W. Kellmann (Rostock, Germany) and T.-R. Soellick (Cologne, Germany).

Construction of amino acid substitution and deletion mutants for two-hybrid analysis. Single and double amino acid substitution mutations were introduced into the coding sequence of the PVY CP core region by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene) and appropriate oligonucleotides according to the manufacturer's instructions. For generating the single amino acid substitutions mutants S125W, R157D, and D201R, the pGEM/T-PVY CP plasmid was used as a template. Double amino acid substitution mutants for PVY CP were obtained by introducing the S125W or D201R mutation, respectively, into the plasmid containing the single mutant R157D or generated by a D201R mutation on the plasmid containing the S125W mutant. After verification of the mutations by sequencing, the mutagenized CP sequences were excised from the pGEM/T cloning vector and introduced into the pGBT9

bait vector. The $\Delta 29N$ and $\Delta 18C$ deletion mutants were generated by PCR amplification of the CP coding region lacking either aa 1 to 29 (nucleotides [nt] 88 to 801 of the PVY CP cDNA sequence) or aa 249 to 267 (nt 1 to 747). PCR fragments were subcloned into pCR-Blunt (Invitrogen) and ligated via EcoRI/Sall restriction sites into pGBT9.

A series of N-terminal deletion mutants from NtCPIP1 and NtCPIP2a were obtained by PCR amplification using appropriate oligonucleotides. EcoRI/Sall-flanked fragments lacking aa 1 to 65 ($\Delta 65N$; nt 246 to 978), 1 to 90 ($\Delta 90N$; nt 331 to 987), and 1 to 115 ($\Delta 115N$; nt 406 to 987) of NtCPIP1 and aa 1 to 66 ($\Delta 66N$; nt 199 to 918), 1 to 94 ($\Delta 94N$; nt 283 to 918), and 1 to 119 ($\Delta 119N$; nt 357 to 918) of NtCPIP2a, respectively, were subcloned into pCR-Blunt and finally introduced into the pAD-GAL4 AD vector (Stratagene).

Screening of phage cDNA library. A λ Zap II cDNA leaf library established from tobacco leaf material (38) was screened with the NtCPIP1 cDNA fragment identified in the yeast two-hybrid screen by standard procedures (38).

Recombinant protein expression and in vitro protein binding assay. To obtain recombinant His₆-tagged NtCPIP proteins, the coding regions of NtCPIP1 and NtCPIP2a were cloned into pQE9 (QIAGEN) by using BamHI and Sall sites, respectively. Both proteins were expressed in *Escherichia coli* M15(pREP4) cells and purified under native conditions using nickel-nitrilotriacetic acid agarose (QIAGEN, Heidelberg, Germany) according to a standard protocol.

For preparation of maltose-binding protein (MBP) fusion proteins, PVY CP encoding cDNA was PCR amplified using appropriate oligonucleotides and inserted as a BamHI/Sall fragment into the pMALc2 vector (New England Biolabs, Beverly, MA). After transformation of the construct into *E. coli* M15(pREP4), recombinant protein expression and cell lysis was performed according to the manufacturer's instructions. The total soluble protein fraction was measured and diluted to 2 μ g of total protein/ μ l, and the portion of the MBP fusion protein was subsequently controlled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For the in vitro binding assay, comparable amounts of MBP fusion protein were incubated for 2.5 h at 4°C with 50 μ l of amylose resin (50% slurry in column buffer [20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM β -mercaptoethanol]) resulting in binding of approximately 130 μ g of protein to the matrix. Amylose-attached MBP samples were transferred to ProbeQuant G-50 microcolumns (Amersham Biosciences, Uppsala, Sweden), washed six times with 750 μ l of column buffer, and incubated with 25 μ g of soluble His₆-tagged NtCPIP1 or NtCPIP2a (diluted to 2 μ g/ μ l in column buffer) for 1 h at room temperature with slight agitation. After the removal of unbound NtCPIP proteins by extensive washing (four times with 700 μ l of column buffer each time), matrix-coupled protein complexes were eluted with 100 μ l of column buffer supplemented with 10 mM maltose. Samples were then subjected to SDS-PAGE and either stained with Coomassie blue as a loading control or blotted onto nitrocellulose membrane (Porablot; Macherey and Nagel, Düren, Germany). Transferred proteins were incubated for 1 h with anti-His monoclonal antibody (diluted 1:3,000; QIAGEN), and immunosignals were detected by chemiluminescence using an anti-mouse horseradish peroxidase-conjugated secondary antibody (diluted 1:100,000) and SuperSignal West Dura extended-duration substrate (Pierce Biotechnology, Rockford, IL).

Binary plasmid construction, agroinfiltration, and preparation of transgenic plants. For transient expression of functional and dominant-negative variants of NtCPIP2a in *N. benthamiana* leaves, full-length (nt 1 to 915) or N-terminally truncated (NtCPIP2a $\Delta 66N$, nt 199 to 915) cDNAs were generated by PCR using appropriate oligonucleotides. BamHI/Sall fragments were inserted into pBinAR (39) downstream of the CaMV 35S promoter and in frame with a C-terminal 3x-Myc epitope. The resulting constructs were introduced into *Agrobacterium tumefaciens* strain C58C1(pGV2260) and infiltrated into the abaxial air space of 4-week-old plants as described previously (69). The p19 protein of *Tomato bushy stunt virus* was used to suppress gene silencing. Coinfiltration of *Agrobacterium* strains containing NtCPIP2a-myc or NtCPIP2a $\Delta 66N$ -myc together with p19 was carried out at an optical density at 600 nm of 1.0:1.0, respectively. Infiltration of p19 alone served as control.

For ectopic expression of dominant-negative mutants of NtCPIP1 and NtCPIP2a, truncated cDNA fragments lacking aa 1 to 65 (NtCPIP1 $\Delta 65N$) and aa 1 to 66 (NtCPIP2a $\Delta 66N$) were amplified by PCR using the gene-specific primers D249 (5'-GGATCCTATATACGGCGATGAGGCGTTGAAATC-3') and D251 (5'-GT CGACTTAGTCAACAGTCTGCCAGCAC-3') for NtCPIP1 $\Delta 65N$ and D250 (5'-GGATCCTGACGTGTACGGTGATGATGCATTG-3') and D210 (5'-GTCTG ACTTAGTCAGCGCTCTGCACAGTAC-3') for NtCPIP2a $\Delta 66N$, respectively. After subcloning into pCR-Blunt, the fragments were inserted into pBinAR between the CaMV 35S promoter and *ocs* terminator. In order to improve translation efficiency, the 5'-untranslated overdrive sequence (Ω) of TMV U1 (32) was placed between the promoter and the NtCPIP1 $\Delta 65N$ or NtCPIP2a $\Delta 66N$ coding sequences, respectively. This also introduced an ATG start codon in an optimized plant con-

sensus sequence within an NcoI cloning site. Stable transformation of tobacco plants with the resulting pBin Ω -NtCPIP1 ΔN_{1-65} and pBin Ω -NtCPIP2a ΔN_{1-66} constructs was performed by *Agrobacterium*-mediated gene transfer as described previously (60).

RNA and protein analysis of transgenic plants. Extraction of total RNA from leaf material and Northern blot analysis was performed as described by Chen et al. (17). NtCPIP1 and NtCPIP2a specific transcripts were detected by using random-primed ³²P-labeled cDNA fragments, respectively.

Protein extraction and Western blot analysis followed the protocol described by Hofius et al. (40). Leaf material was homogenized in 2 $\times$ SDS sample buffer containing 50 mM Tris-HCl, 5% (vol/vol) β -mercaptoethanol, 10% (vol/vol) glycerol, and 2% (wt/vol) SDS (pH 6.8). After heat denaturation, equal amounts of protein were separated on 12.5% (vol/vol) SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Porablot). An immunoreaction was carried out with either rabbit polyclonal anti-Myc antibody (1:3,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or polyclonal anti-NtCPIP1 (1:3,000) and anti-NtCPIP2a antiserum (1:5,000), generated against affinity-purified His₆-tagged NtCPIP1 and NtCPIP2a proteins (see above) in rabbits using custom service from Eurogentec (Seraing, Belgium).

BiFC assay. Protein-protein interaction studies using the BiFC technique were carried out as described previously (72). For construction of the binary plasmids, full-length cDNAs of PVY CP and both NtCPIPs were PCR amplified using appropriate oligonucleotides and fused as BamHI/Sall fragments to either the N-terminal portion of YFP (YFP^N) in the binary vector pSPYNE-35S (NtCPIP1 and NtCPIP2a) or to the C-terminal part of yellow fluorescent protein (YFP^C) in pSPYCE-35S (PVY CP). The resulting constructs were introduced into *A. tumefaciens* strain C58C1(pGV2260) and agroinfiltrated in pairwise combinations together with the p19 silencing suppressor (optical density at 600 nm of 1.0:1.0:1.0) into leaves of 3-week-old *N. benthamiana* plants according to the procedure described above. Three days after infiltration, coexpression of proteins was assured by Western blot analysis with anti-HA and anti-Myc antibodies (data not shown). For microscopic analysis, sections from agroinfiltrated leaves were manually cut, incubated in 50 mM phosphate buffer (pH 7.2), and scanned in the epidermal cell layer for reconstituted YFP fluorescence by using the confocal microscope LSM 510 META (Zeiss, Göttingen, Germany). An excitation light of 488 nm produced by the krypton/argon laser and an emission filter of 516 to 537 nm allowed the detection of YFP-specific fluorescence, which was finally superimposed with the Nomarski scan by means of the Zeiss LSM version 3.0.

Virus infections and movement studies. PVY infection of tobacco plants and immunological detection of PVY CP was performed as described previously (37) using virus-specific enzyme-linked immunosorbent assay (ELISA) reagents provided by BIOREBA (Reinach, Switzerland).

For analysis of viral movement, PVY was tagged with the green fluorescent protein (GFP) by using the intron stabilized PVY-123 full-length cDNA clone (11; kindly provided by E. Johansen, University of Copenhagen, before publication) for inserting smRS-GFP (18) according to the same cloning strategy as published by Dietrich and Maiss (20). The full-length clone was propagated in *E. coli* NM522 (Pharmacia), and DNA was prepared by using a QIAGEN maxikit (QIAGEN). Biolistic delivery of the GFP-labeled PVY cDNA into tobacco leaves was performed with a hand-held particle gun (Bio-Rad Helios gene gun system). Detection and visualization of initial infection sites was achieved by bombardment of the GFP-tagged PVY cDNA together with a DsRed expression vector (pe35AsclOptRed) (21). Fluorescently tagged virus was imaged by confocal laser scanning microscopy (Leica TCS SP2) 4 days postinfection (dpi). GFP and DsRed were excited with the argon laser (488 nm) and the helium-neon laser (543 nm), respectively. Cross talk was eliminated by previous lambda-scanning or manual regulation of laser intensity.

Sequence data from the present study have been deposited with the EMBL/GenBank data libraries under accession numbers AY319647 (PVY CP), AY319648 (NtCPIP1), AY319649 (NtCPIP2a), AY319650 (NtCPIP2b), respectively.

RESULTS

Isolation of PVY CP interacting tobacco proteins. To identify plant proteins that interact with PVY CP, the GAL4-based yeast two-hybrid system (30) was used. The entire PVY CP coding region was amplified by reverse transcription-PCR from PVY^N (N-strain)-infected leaf material (37) and used in fusion with the GAL4 BD as bait to screen an AD-tagged

cDNA library constructed from tobacco source leaves. Out of a total number of approximately 8×10^7 transformants, four positive clones were identified showing activity of both reporter genes *HIS3* and *lacZ*. Library plasmids were rescued, and the specificity of the interaction was verified by retransformation into the reporter strain in combination with the bait BD-PVY CP or control plasmids (Fig. 1A). Sequence analysis revealed that the isolated cDNA clones encoded for an identical protein designated NtCPIP1. Among these clones, the longest AD-fused open reading frame (ORF) coded for 270 aa. The full-length NtCPIP1 cDNA clone encoding a protein of 306 aa was obtained by screening a cDNA tobacco leaf library (38) using the *NtCPIP1* cDNA fragment as a probe. Remarkably, two additional cDNA clones different from *NtCPIP1* but highly similar to each other were isolated and subsequently designated *NtCPIP2a* and *NtCPIP2b*. Alignment of the protein sequences showed that NtCPIP1 shared substantial similarity of 81.3 and 82.6% identical amino acid residues with NtCPIP2a and NtCPIP2b, respectively, whereas NtCPIP2a and NtCPIP2b were 97.4% identical to each other (Fig. 1B). Yeast two-hybrid analyses with AD-tagged constructs of NtCPIP2a and NtCPIP2b in combination with BD-PVY CP (Fig. 1C) or appropriate control plasmids (data not shown) verified a similar binding of NtCPIP2a and NtCPIP2b to PVY CP as shown for NtCPIP1. Due to the presence of a conserved J-domain (44) at the N terminus (aa 4 to 68 for NtCPIP1 and aa 4 to 70 for NtCPIP2a/2b, respectively), the CP-interacting NtCPIPs could be assigned to the large and diverse family of DnaJ-like proteins, and highest similarity was detected to several DnaJ-like genes from *Arabidopsis thaliana* (GenBank accession numbers AAD39315, AAS32885, AAF07844, AAD25656, and T48181). Interestingly, NtCPIPs also shared substantial similarity with a subset of J-domain proteins encoded by the *N. tabacum* gene NtDnaJ_M541 (59.2% identity on amino acid level) and its *Lycopersicon esculentum* Le19/8 (59.5%) and *Arabidopsis thaliana* AtA39 (59.8%) orthologues that were previously identified to interact with the TSWV movement protein NSm (66, 70). However, PVY CP was unable to bind NtDnaJ_M541 and consistently TSWV NSm did not interact with the different NtCPIPs in yeast (Fig. 1C).

Mutations in the core region of PVY CP abolish yeast interaction with NtCPIPs. To analyze whether the interaction between PVY CP and NtCPIPs can be mapped either to the core region or to the N- and C-terminal regions of the CP, a series of PVY CP mutants as GAL4-binding domain fusions were generated. To this end, N-terminal (29 aa, PVY CPΔ29N) and C-terminal (18 aa, PVY CPΔ18C) deletions were made or single and double amino acid substitutions were generated by site-directed mutagenesis. The amino acids targeted in these substitution mutants were three highly conserved residues in the core region of potyviral CPs (25), which have been demonstrated to be essential for successful cell-to-cell movement of TEV (23, 24), as well as for PD-mediated trafficking of pressure-injected recombinant *Bean common mosaic necrosis virus* and *Lettuce mosaic virus* CPs (59). Qualitative yeast two-hybrid assays were performed with the single amino acid substitutions PVY CP S125W, PVY CP R157W, and PVY CP D201R, as well as the double amino acid substitutions PVY CP S125W/R157D, PVY CP S125W/D201R, and PVY CP R157D/D201R (Fig. 2). Binding of PVY CP to

FIG. 2. Identification of the PVY CP interaction domain in the yeast two-hybrid system. PVY CP deletion and various single and double amino acid substitution mutants were individually cotransformed with NtCPIP1 or NtCPIP2a into yeast cells and qualitatively assayed for β -galactosidase activity. Mutations introduced into the PVY CP gene are indicated. Amino acids (S125, R157, and D201) targeted in the substitution mutants refer to highly conserved residues in the core domain of potyviral CPs (22).

NtCPIP1, as well as to NtCPIP2a, was not influenced by N- and C-terminal deletions; however, *lacZ* reporter gene expression was completely abolished by the three single amino acid substitutions and all combinations of double amino acid substitution mutants, suggesting an essential role of the CP core region for interaction with NtCPIPs.

NtCPIPs interact with PVY CP in vitro and in planta. To confirm the interaction ability of NtCPIPs with PVY CPs, an independent in vitro protein binding assay was used. To this end, PVY CP was expressed as MBP fusion in *E. coli* cells and coupled to an amylose affinity matrix. After incubation with either His-tagged NtCPIP1 or NtCPIP2a proteins, matrix-bound protein complexes were eluted and subsequently analyzed for the presence of NtCPIPs by Western blotting with anti-His antibodies. In agreement with the results obtained by the two-hybrid analysis, NtCPIP1 (Fig. 3A) and NtCPIP2a (Fig. 3B) showed binding to PVY CP, whereas MBP alone did not interact.

To verify the association of PVY CP with NtCPIPs in planta, a BiFC assay was performed in *Agrobacterium*-infiltrated *N. benthamiana* plants (72). NtCPIP1 and NtCPIP2a were fused to the N-terminal YFP fragment (YFP^N), respectively, whereas PVY CP was merged with the C-terminal YFP fragment (YFP^C). Pairwise expression of unfused YFP^N and YFP^C or their combination with the respective PVY CP or NtCPIP fusions induced very weak or no YFP fluorescence signals in agroinfiltrated epidermal cells (Fig. 3C to F). In contrast, strong YFP fluorescence was observed when combinations of PVY CP and NtCPIP1 or NtCPIP2a were expressed, respectively, indicating the capability of PVY CP/NtCPIP complex formation in plant cells (Fig. 3G and H).

Ectopic expression of dominant-negative mutants of NtCPIPs confers resistance to PVY. To assess the in planta role of NtCPIP binding to PVY CP during virus infection, we sought to generate transgenic plants with impaired NtCPIP function. An obvious approach was the downregulation of NtCPIPs via posttranscriptional gene silencing by stable expression of hair-

FIG. 3. PVY CP interacts with NtCPIPs in vitro and in planta. (A and B) In vitro binding assay between PVY CP and NtCPIP1 (A) or NtCPIP2a (B). MBP alone (lanes 1 and 4) or in fusion with PVY CP (lanes 2 and 5) was expressed in *E. coli*, coupled to an amylose matrix, and incubated with 25 μ g of affinity-purified His₆-tagged NtCPIP1 or

pin RNA interference (RNAi) constructs (65). However, such a strategy might be limited by an insufficient degree of suppression, the functional complementation of RNAi-silenced NtCPIPs by unknown and nontargeted DnaJ isoforms, and/or the reversion of silencing by the potent potyviral silencing suppressor HC-Pro after virus infection (9, 61). Indeed, infection of transgenic plants specifically silenced for either NtCPIP1 or NtCPIP2a revealed an enhanced local but only transient resistance to PVY (data not shown).

To circumvent such potential constraints of a silencing approach, we intended to ectopically express dominant interfering variants of NtCPIP proteins in transgenic plants. These mutants should retain their ability to bind to PVY CP but should be unable to serve their cellular function. Generally, DnaJ proteins are assumed to function as cochaperones and regulators of heat shock protein 70 (HSP70) proteins by stimulating their ATPase activity via interaction of the J domain (44). Therefore, we analyzed in the yeast two-hybrid system whether deletion of the main portion of the J domain from NtCPIP1 and NtCPIP2a would also affect the interaction with PVY CP. N-terminal deletions of aa 1 to 65 and aa 1 to 66 of NtCPIP1 and NtCPIP2a, respectively, did not abolish *lacZ* reporter gene activity, suggesting that the J domain is dispensable for binding to PVY CP. However, extending the N-terminal truncations to more than 90 aa resulted in a complete loss of binding (Fig. 4A).

To test the effect of N-terminally deleted NtCPIP mutant versus the full-length protein on PVY spread in planta, we combined agroinfiltration-mediated protein expression of NtCPIP2a variants with a virus infection assay in *N. benthamiana* leaves. To this end, full-length NtCPIP2a and J-domain-deficient NtCPIP2a Δ N cDNA fragments were placed into a binary vector between the constitutive CaMV 35S promoter and a 3x-Myc epitope tag. The resulting constructs were coinfiltrated with the silencing suppressor p19 into *N. benthamiana* leaves, leading to considerable protein expression until 4 to 6 days postinfiltration (Fig. 4B). Leaves were challenged with PVY 24 h after agroinfiltration and assayed for virus accumulation at 4 dpi by using an ELISA. As demonstrated in Fig. 4C, only expression of NtCPIP2a Δ N and not of full-length NtCPIP2a strongly affected the establishment of virus infection

NtCPIP2a protein. Aliquots of the eluates (75% of total amount) were separated by SDS-PAGE and tested for the presence of NtCPIPs (arrowheads) by Coomassie blue staining or Western blot analysis with anti-His antibodies. Then, 2 μ g of His₆-NtCPIP1 (lane 3) or His₆-NtCPIP2a (lane 6) was loaded onto the respective gels as input controls. (C to H) BiFC analysis of PVYCP/NtCPIP interaction in plant cells. The coding regions of NtCPIPs and PVY CP were fused with the N-terminal (YFP^N, in pSPYNE-35S) or C-terminal (YFP^C, in SPYCE-35S) region of YFP, respectively. Plasmids were *Agrobacterium* infiltrated in *N. benthamiana* leaves, and the reconstructed YFP signal was detected in the epidermal cell layer by confocal microscopy. Coexpression of empty vectors pSPYNE/pSPYCE (C), PVY CP:YFP^C/pSPYNE (D), pSPYCE/NtCPIP1:YFP^N (E), pSPYCE/NtCPIP2a:YFP^N (F) PVY CP:YFP^C/NtCPIP1:YFP^N (G), or PVY CP:YFP^C/NtCPIP2a:YFP^N (H) reveals specific YFP complementation only by PVY CP/NtCPIP interactions. YFP-derived fluorescence signals (in green) of single confocal sections (left) and the transmission mode (middle) were superimposed in the merged image (right). Bars, 50 μ m.

FIG. 4. Identification of dominant-negative NtCPIP mutants and functional analysis in planta. (A) Interaction of N-terminal deletion mutants of NtCPIP1 and NtCPIP2a with PVY CP in the yeast two-hybrid system. Yeast cells cotransformed with the indicated bait and prey plasmids were grown on Trp⁻/Leu⁻/His⁻ medium and qualitatively assayed for β -galactosidase activity. (B) Transient expression of 3x-Myc epitope-tagged full-length and J-domain-deficient NtCPIP2a proteins in *N. benthamiana* leaves via agroinfiltration. Western blot analysis was performed with identical amounts of total protein extracts from leaves 0, 4, and 6 days after coinfiltration of NtCPIP2a-myc and NtCPIP2aΔN-myc with the p19 silencing suppressor, respectively. Infiltration of p19 alone served as a negative control. (C) Effect of transient expression of full-length and J-domain-deficient NtCPIP2a proteins on susceptibility to PVY infection in *N. benthamiana*. Leaves were infected with PVY 24 h after agroinfiltration and assayed for accumulation of viral coat protein 4 dpi by ELISA. Values represent means ($n = 12$) $\pm$ the standard error (SE) and are given as the percentage of the p19 control. The results indicate that expression of the dominant-negative mutant but not the full-length variant strongly interferes with the spread of infection.

in the local leaf, supporting the concept that J-domain-deleted but CP-interacting NtCPIP variants function as dominant-interfering mutants in planta.

Based on these results, binary constructs for stable expression of dominant-negative variants both for NtCPIP1 and for NtCPIP2a were generated. In order to reach a preferably high expression level in transgenic plants, N-terminal deleted fragments of NtCPIPs were fused to the translational enhancer (Ω) from TMV U1 (32) downstream of the CaMV 35S promoter, resulting in the plasmids pBin Ω -NtCPIP1 Δ N₁₋₆₅ and pBin Ω -NtCPIP2a Δ N₁₋₆₆ (Fig. 5A). After *Agrobacterium*-mediated transformation, 26 primary transformants for each construct were transferred to the greenhouse and screened for expression of the transgene by Northern analysis. Several plants accumulating considerable amounts of the respective transcripts could be identified (data not shown). Two plants bearing the

construct pBin Ω -NtCPIP1 Δ N₁₋₆₅ (designated NtCPIP1 Δ N-9 and -17) and four plants transgenic for the pBin Ω -NtCPIP2a Δ N₁₋₆₆ construct (NtCPIP2a Δ N-15, -16, -28, and -39) were chosen for further analysis (Fig. 5B). To verify the accumulation of J-domain truncated NtCPIP proteins, leaf samples of the selected lines were subjected to Western analysis with either NtCPIP1- or NtCPIP2a-specific antibodies. As demonstrated in Fig. 5C, transgenic plants accumulated high amounts of the dominant-negative NtCPIP variants, which, however, did not result in detectable morphological changes or growth defects compared to wild-type (WT) or transgenic controls (data not shown).

To investigate whether accumulation of the truncated NtCPIP1 and NtCPIP2a mutant proteins would interfere with PVY multiplication and systemic spread, the kanamycin-resistant T1 progeny of the selected transgenic plants were challenged with PVY and compared to the wild type and a transgenic control (ME). A total of 22 to 25 plants of the candidate lines, as well as a similar number of WT ($n = 22$) and ME ($n = 24$) plants were mechanically inoculated at the 7-leaf-stage, and the development of virus infection was monitored by visual symptom analysis and immunological determination of coat protein levels. All inoculated individuals of the transgenic (ME) and nontransgenic control lines (WT) started to display typical disease symptoms in systemic leaves at 5 to 6 dpi, whereas the different transgenic lines did not show any visible virus-induced symptoms at this time point (data not shown). Consistently, the virus titer in systemic leaves was strongly reduced to almost complete resistance in the transgenic lines (Fig. 5D). The durable increase in virus resistance was confirmed at 13 dpi, where all transgenic lines showed dramatically reduced or absent viral symptoms in comparison to the control lines (Fig. 5F). Analysis of the virus titer in systemic leaves verified the strongly reduced systemic invasion by PVY, which was most pronounced in line NtCPIP2a Δ N-39 (Fig. 5E). However, viral particles were detectable in systemic leaves of all transgenic lines, indicating that the resistance conferred by expression of dominant-negative NtCPIP mutants did not provide immunity to PVY infection.

To exclude the possibility that the increased virus resistance was caused by interference of the TMV-derived Ω sequence, transgenic plants expressing an unrelated viral protein (HPV L1 CP) under control of the CaMV 35 promoter and the identical Ω translation enhancer (6) were challenged with PVY and compared to WT and NtCPIP Δ N transgenic lines. Determination of virus titer in systemic leaves at 6 dpi confirmed a strong reduction of virus spread in NtCPIP Δ N plants, which was not seen in L1 transgenic and WT plants, indicating no major impact of the Ω sequence on resistance parameters (data not shown).

Expression of dominant-negative NtCPIP mutants impairs the local spread of PVY-gfp. Our finding that mutations in the core region of CP abolished the binding to NtCPIPs in the yeast system suggested the potential contribution of PVY CP-NtCPIP interaction to virion assembly and cell-to-cell movement rather than to long-distance transport. Therefore, we tested whether the NtCPIP Δ N transgenic lines were affected in viral cellular spread by using a GFP-labeled PVY cDNA clone that was delivered into epidermal cells of fully expanded source leaves via particle bombardment. As shown in Fig. 6A and B, WT and transgenic control

FIG. 5. Effect of stable expression of dominant-negative NtCPIP mutants on susceptibility to PVY infection. (A) Schematic representation of binary overexpression constructs used for transformation of *N. tabacum*. J-domain-deficient NtCPIP1 Δ 65N and NtCPIP2a Δ 66N fragments shown to retain their interaction ability with PVY CP (Fig. 4A) were fused to the 5'-untranslated TMV U1 overdrive sequence (Ω) and placed between the CaMV 35S promoter and ocs terminator in the Bin19-derived vector. (B) Northern analysis of NtCPIP1 Δ 65N and NtCPIP2a Δ 66N specific transcripts. Each lane contains 30 μ g of total RNA isolated from WT plants and transgenic lines NtCPIP1 Δ -9 and -17 and NtCPIP2a Δ -15, -16, -28, and -39. Northern blots were hybridized with NtCPIP1 Δ 65N or NtCPIP2a Δ 66N cDNAs, respectively. (C) Immunoblot analysis of NtCPIP1 Δ 65N and NtCPIP2a Δ 66N protein accumulation. Identical amounts of total protein extracted from leaf material of WT and transgenic lines were separated by SDS-PAGE and analyzed by Western blotting with rabbit-derived polyclonal anti-NtCPIP1 (dilution 1:3,000) or anti-NtCPIP2a (1:5,000) antibodies and goat-derived secondary antibody conjugated to horseradish peroxidase (dilution 1:100,000). (D) PVY titer in systemic leaves (five or six leaves above the inoculated leaf) of WT ($n = 22$) and transgenic ME-4 controls (ME, $n = 24$), as well as of the transgenic lines NtCPIP1 Δ -9 ($n = 25$) and -17 ($n = 25$) and NtCPIP2a Δ -15 ($n = 23$), -16 ($n = 24$), -28 ($n = 24$), and -39 ($n = 24$) at 6 dpi. Values represent means $\pm$ the SE and are given as the percentage of the WT level. Plants had developed six to eight leaves prior to PVY inoculation. (E) PVY coat protein levels in systemic leaves (seven or eight leaves above the inoculated leaf) of WT and transgenic lines at 13 dpi. Values represent means $\pm$ the SE and are given as the percentage of the WT level. (F) Development of virus-induced symptoms in PVY-infected transgenic lines compared to controls (WT, ME) at 13 dpi, indicating a dramatically increased virus resistance due to the expression of dominant-negative mutants of NtCPIPs.

FIG. 6. Effect of dominant-negative NtCPIP mutants on cellular spread of PVY-gfp. PVY-gfp was cobombarded with a DsRed expression vector into leaves of WT (A) and transgenic (B) controls, as well as leaves of the transgenic lines NtCPIP1ΔN-9 (C) and NtCPIP2aΔN-39 (D). At 4 dpi, bombarded leaves were scanned for GFP- and DsRed-derived fluorescence by confocal microscopy. Representative lesions demonstrated that infections in transgenic lines NtCPIP1ΔN-9 and NtCPIP2aΔN-39 reached a considerably smaller area beyond the primarily bombarded cells (indicated in red) than in the control lines. Bars, 100 μ m.

(ME) plants showed uniform GFP fluorescence in initial infection loci 4 days after bombardment that reached an area up to three cells beyond the primarily bombarded cell (visualized by red fluorescence derived from expression of the cobombarded CaMV 35S-DsRed plasmid). In contrast, transgenic lines NtCPIP1ΔN-9 and NtCPIP2aΔN-39 showed considerably smaller zones of GFP fluorescence, which reached only approximately one to two cells in diameter beyond the primary target cell (Fig. 6C and D). These results suggested that impaired NtCPIP function primarily affected viral cell-to-cell transport, thereby causing an overall increase in resistance to PVY infection.

DISCUSSION

In many aspects, cell-to-cell spread of potyviruses still remains an enigma because the intercellular transfer of viral genomes is not mediated by specialized MPs but relies on various proteins with multifunctional properties (57, 67). The CP has been suggested to be implicated in cell-to-cell movement due to its requirement for virion formation and the capacity to traffic viral RNA through modified PD into adjacent cells (24, 59). However, the molecular pathway by which potyviral CPs contribute to intra- and intercellular transport of infectious material is largely unknown. To unravel these processes, it would be necessary to isolate plant proteins that directly interact with the CP in the host cell during infection. Here, we have performed a yeast two-hybrid assay by using PVY CP as bait resulting in the isolation of a subset of DnaJ-like proteins from tobacco, termed NtCPIPs, which were dem-

onstrated to specifically bind to PVY CP in yeast, in vitro and in planta. The functional significance of the interaction could be verified by virus infection of agroinfiltrated *N. benthamiana* leaves or transgenic plants that ectopically overexpressed dominant-negative mutants of NtCPIPs. These plants showed significantly enhanced virus resistance to PVY, most likely due to strongly reduced cell-to-cell transport. Thus, it can be concluded that recruitment of host chaperones is required for efficient spread of PVY, presumably by interaction of the viral CP with a specific subset of plant DnaJ-like proteins.

Members of the DnaJ (HSP40) multigene family are generally defined by the presence of a N-terminally located J domain and assist as cochaperones of HSP70s in various cellular processes such as protein folding, trafficking, and secretion and also in stress response signaling (10, 44, 50, 56, 71). DnaJ proteins are structurally diverse and grouped according to the combination of three additional domains initially identified in the *E. coli* DnaJ ortholog: a Gly/Phe-rich domain, a Cys-rich zinc finger domain and a less well conserved C-terminal domain possibly involved in substrate binding (56). However, the identified NtCPIPs fall into a distinct subclass that contain only the J domain. Strikingly, the group of DnaJ-like proteins from tobacco (NtDnaJ_M541), tomato (Le19/8) and *Arabidopsis* (AtA39), which were recently isolated as TSWV MP (NSm)-binding proteins, also lack, except for the common J domain, domain structures typically found in DnaJ (66, 70). Instead, motifs for a Lys-rich domain (4x K-X-X-X-K-E/K) were identified that are also partially present in the NtCPIP proteins (aa 172 to 177 and aa 269 to 274 for NtCPIP1 and aa 171 to 176 and aa 168 to 273 for NtCPIP2a and -2b, Fig. 1B). However, despite significant sequence and structural similarities, NtCPIPs and NtDnaJ_M541 were not interchangeable for interaction with PVY CP and TSWV MP (NSm), respectively (Fig. 1C), suggesting some specificity of viral proteins in their interaction capability with members of this DnaJ subclass. Independent binding assays verified the ability of PVY CP/NtCPIP complex formation in vitro and in planta (Fig. 3), which significantly strengthened the likelihood that these DnaJ-like protein family members indeed represent novel and relevant binding partners of the potyviral CP.

Unequivocal evidence for a critical role of NtCPIPs in potyviral pathogenesis was provided by resistance analysis of transgenic tobacco plants impaired in NtCPIP function due to overexpression of dominant interfering variants of NtCPIP1 and NtCPIP2a. These deletion mutants lacked the main portion of the J domain which is required for interaction with cellular HSP70 proteins (44) but is dispensable for association with the PVY CP, as revealed by yeast two-hybrid analysis (Fig. 4A). Initially, proof of concept for the inhibitory effect of J-domain deletion mutants versus the full-length protein on PVY infection could be obtained by a combined agroinfiltration and infection assay in *N. benthamiana* leaves (Fig. 4B). Local PVY accumulation was severely inhibited by transient expression of J-domain-deficient but not of full-length NtCPIP2a (Fig. 4C), suggesting the requirement of NtCPIP function during the initial phases of virus infection. Indeed, mutant analysis in the yeast two-hybrid system identified the CP core region as an essential interaction domain (Fig. 2), thereby linking the PVY CP/NtCPIP association to assembly and plasmodesmal trafficking rather than to long-distance

transport processes. Circumstantial evidence for this notion was additionally provided by movement studies using biolistically delivered PVY-gfp, which showed strongly delayed spreading from the primarily infected cell in NtCPIP1 Δ N-9 and NtCPIP2 Δ N-39 lines compared to the WT and transgenic controls (Fig. 6). Hence, the dominant-negative NtCPIP proteins might have primarily interfered with cell-to-cell transport processes, which finally resulted in strongly enhanced and durable resistance of various independent NtCPIP Δ N expressing lines to PVY infection (Fig. 5D to F). Our observation that the increased resistance did not provide immunity to PVY might indicate that the obtained expression level of dominant-negative mutants did not completely suppress the functioning of endogenous NtCPIPs. Nonetheless, the movement and resistance data clearly demonstrate the *in vivo* relevance of the interaction between the CP and host proteins from the DnaJ family and thus suggest the involvement of HSP70-related mechanisms in PVY infection.

The previously observed binding of the TSWV NSm to the NtCPIP related J-domain proteins from different plant species indicate that different plant viruses might have evolved a similar strategy to exploit HSP70-related chaperone activity in various virulence functions (66). However, in contrast to the present study, experimental data are still lacking demonstrating the *in planta* role of the NSm-DnaJ protein interaction during TSWV infection. Thus, direct evidence for the importance of HSP70 class proteins in plant virus infection has thus far only been provided by the family of closteroviruses, which encode the only known virus-specific HSP70 homologs (HSP70h) (1). The HSP70h of beet yellows virus has been shown to associate with PD (51) and to function as one of the closteroviral MPs (55). In addition, HSP70h was demonstrated to be essential for virion assembly and stability (2). Interestingly, the basic morphology of the filamentous virions of closteroviruses seems to be similar to that of potyviruses and some other plant virus genera. Accordingly, capsid proteins of these viruses are structurally and evolutionarily related to each other (25). However, closteroviruses are exceptionally long, a feature which was suggested to be the reason for the evolution of two specialized CPs and the integration of HSP70h into the viral genome, thereby providing additional energy for assembly and translocation (2). Due to the dual role of HSP70h in closteroviral virion formation and transport, as well as its concerted action with CPs, it is tempting to speculate that potyviruses may have adopted a similar movement strategy by incorporation of host cellular HSP70s into or in association with the potyviral transport complex. Interestingly, a subclass of plant HSP70 proteins from *Cucurbita maxima* was previously identified showing plasmodesmal targeting and translocation capacity (3). Members of this subclass might be potential HSP70 candidates recruited by the binding of host NtCPIPs to viral CP and assumed to be required for the chaperone-aided transport of virus particles toward and through PD. A major challenge for the future will be to identify those HSP70 proteins which are able to interact with NtCPIPs.

In summary, our work identifies NtCPIPs as novel potyviral susceptibility factors and also provides a strong *in vivo* confirmation for the essential role of plant chaperones in virus movement. Taking this into account, the recruitment of molecular

chaperones is emerging as a widespread mechanism by which certain plant viruses conquer the host.

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