Cell-Penetrating Peptides Derived from Viral Capsid Proteins

Xiaopeng Qi, Tyler Droste, and C. Cheng Kao

Department of Molecular & Cellular Biochemistry, Indiana University, Bloomington, IN 47405, U.S.A.

Submitted 1 July 2010. Accepted 8 August 2010.

Cell-penetrating peptides (CPP) can translocate across the cell membrane and have been extensively studied for the delivery of proteins, nucleic acids, and therapeutics in mammalian cells. However, characterizations of CPP in plants have only recently been initiated. We showed that the intact virion and a recombinant capsid protein (CaP) from a plant-infecting nonenveloped icosahedral RNA virus, *Brome mosaic virus* (BMV), can penetrate the membranes of plant protoplasts but are trapped by the extracellular matrix. Furthermore, a 22-residue peptide derived from the N-terminal region of the CaP (CPNT) can enter barley protoplasts and cells of intact barley and *Arabidopsis* roots. An inhibitor of the macropinocytosis reduced CPNT entry, while treatment with NiCl_2 changed the cellular localization of CPNT. CPNT increased uptake of the green fluorescent protein (GFP) into the cell when covalently fused to GFP or when present in trans of GFP. The BMV CPNT overlaps with the sequence known to bind BMV RNA, and it can deliver BMV RNAs into cells, resulting in viral replication, as well as deliver double-stranded RNAs that can induce gene silencing.

Cell-penetrating peptides (CPP) have the ability to translocate across the cell membrane and effect changes in cells. The Tat protein from *Human immunodeficiency virus 1* (HIV) was first identified to possess a CPP motif (Frankel and Pabo 1988; Green and Loewenstein 1988). Subsequent studies found that an arginine-rich motif (ARM) in Tat was required to translocate across cell membranes (Futaki et al. 2001). CPP that function in animal cells are classified to be cationic (e.g., Tat, penetratin, pVEC, and polyarginine) or amphipathic (e.g., transportan and mitogen-activated protein) (Derossi et al. 1994; Elmquist et al. 2001; Futaki et al. 2001; Oehlke et al. 1998; Pooga et al. 1998). CPP can have reduced immunogenicity and cytotoxicity, features that are attractive for drug delivery and other therapeutic purposes (Fernandez-Carnead et al. 2004).

The mechanism of CPP internalization and intracellular trafficking in mammalian cells remains somewhat controversial. Some reports indicate that CPP uptake occurs by the macropinocytosis and endocytosis pathways, while others suggest that receptor-mediated and energy-independent nonendocytotic mechanisms are used (Drin et al. 2003; Jones 2007; Mano et

al. 2005). In addition, the initiation of CPP uptake has been reported to require a Rac1-GTPase-dependent actin network and also membrane-associated proteoglycans (Gerbal-Chaloin et al. 2007; Nakase et al. 2007).

Pathogens may use protein transduction mediated by CPP motifs to gain entry into animal cells (Ford et al. 2001). Recently, an arginine-rich peptide derived from the capsid protein (CaP) of *Flock house virus* was shown to be internalized into mammalian cells via macropinocytosis to mostly accumulate in membranes (Nakase et al. 2009). Vaccinia virus, an enveloped DNA virus with a dimension in excess of 250 nm, has also been reported to enter cells by several pathways, including macropinocytosis (Mercer and Helenius 2008; Smith and Helenius 2004). For plant viruses, the entry mechanism is poorly understood. Textbooks usually state that plant viruses do not use receptor-mediated entry but require a physical breach of the plant cell wall. If the plasma membrane remained intact, there should be a mechanism for the viruses to pass through this and other membranes. Arginine-rich peptides have been reported to enter tobacco or triticale protoplast and maize root-tip cells (Chang et al. 2007; Chugh et al. 2009; Mäe et al. 2005). Furthermore, *Rice dwarf virus*, which also has an insect host, can enter insect cells through mechanisms similar to those of mammalian viruses (Wei et al. 2007). Ectopic expression of the CaP from *Rice dwarf virus* in insect cells can induce membrane fusion (Zhou et al. 2007).

We are interested in the regulatory roles of the CaP of the monocot-infecting *Brome mosaic virus* (BMV), a positive-stranded tripartite RNA virus that has served as a model system, because it can replicate in several hosts, including yeast (Janda and Ahlquist 1993; Kao and Sivakumaran 2001; Noueiry and Ahlquist 2003). BMV packages its three genomic RNAs into separate nonenveloped icosahedral particles that contain 180 subunits of the CaP.

The approximately 40 N-terminal residues of the CaP, also known as the N-terminal tail, participate in encapsidation of the BMV genomic RNA (Duggal and Hall 1993; Sacher and Ahlquist 1989). Residues 9 to 22 of the CaP contain an ARM that is sufficient to bind to the BMV RNA (Sgro et al. 1986). Futaki and associates (2001) noted that it resembles a CPP. Since the N-terminal tail exists inside the virion, it must either be extruded from the viral particle or function when the virion is partially dissociated to act to transduce proteins. In the crystal structure of the virion, the N-terminal tails are poorly resolved and the one complete N-terminus in an asymmetric trimer was not visualized to be in direct contact with the RNA that lay underneath the capsid (Lucas et al. 2002). Intriguingly, the comparable sequence in the closely related Bromovirus *Cowpea chlorotic mottle virus* (CCMV) is among the very first cleaved by trypsin when the virion undergoes structural transition prior to the release of the viral RNA (Liepold et al. 2005).

Current address for X. Qi: Department of Medicine and Integrated Department of Immunology, National Jewish Hospital, Denver, CO 80206, U.S.A.

Corresponding author: C. Cheng Kao;
Telephone: +1.812.856.5710; E-mail: ckao@indiana.edu

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary tables and two supplementary figures are published online.

A deletion of this ARM in the CCMV CaP resulted in a defect in the systemic spread of the virus (Annamalai et al. 2005). In this study, we characterized a portion of the N-terminal tail as well as the BMV CaP for their abilities to enter plant cells and to deliver proteins and RNAs.

RESULTS

BMV entry into barley protoplast.

We first determined whether the intact BMV virion has the ability to cross the membranes of protoplasts derived from barley leaves (Fig. 1A). Purified virions were added to solutions of barley protoplasts for between 5 min to 12 h in the absence of polyethylene glycol (PEG) or Ca^{2+} , followed by harvesting of the protoplasts by three rounds of centrifugation and washing. The protoplasts were then suspended in a lysis buffer and were subjected to Western blot analysis. Protoplast-associated CaP signal was detected within 5 min of adding the virions and increased over time, with detectable increase by 40 min. (Fig. 1A). This timing for the increase should precede the replication, subgenomic transcription of RNA4, and translation of the CaP (Sivakumaran et al. 2005). It is possible that accumulations of the CaP observed several hours later could be due to gene expression from BMV RNA.

To visualize the BMV virion entry into protoplast, we made a fluorescently labeled BMV. The 6-amino acid sequence (CCPGCC) that can bind the biarsenic fluorescein dye FIAsh was inserted to the N-terminal tail of the BMV CaP between residues 23 and 24 (Fig. 1B). This position was selected because it was distinct from the arginine-rich sequence that binds RNA and the globular portion of the CaP that is involved in subunit interactions (Fig. 1B). The construct was infiltrated into *Nicotiana benthamiana* along with *Agrobacterium* cultures that can express BMV RNA1 and RNA2, and the leaves were collected for virion preparations after 8 days. The virus with the insertion will be named fBMV. In three independent preparations, fBMV was obtained at approximately half of the yield of wild-type BMV after purification through a cesium chloride gradient (data not shown). The fBMV particles had morphology highly similar to that of wild-type BMV and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified virions revealed that the protein has a slight increase in molecular mass and also a higher abundance of dimers even in denaturing and reduced gels when compared with the wild type (WT). This could indicate that this portion of the BMV CaP could interact with each other to a greater extent than that of the WT virion.

Fig. 1. Brome mosaic virus (BMV) virus entry into protoplasts. **A**, Detection of BMV virion entry into barley protoplasts as a function of time. The BMV capsid protein (CaP) was detected within protoplasts by Western blot using a specific polyclonal antibody. **B**, A fluorescent BMV virion. The sequence of the N-terminal portion of the BMV CaP and the location of the FIAsh dye-binding sequence (CCPGCC, in bold letters). Within the box is a model of the BMV CaP monomer and the approximate location of the inserted FIAsh dye-binding sequence. The mutant virus will be named fBMV. The gel image illustrates the proteins from wild-type (WT) BMV CaP and fBMV virions were purified from a cesium chloride density gradient. The virions were denatured in Laemmli sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The monomers of the fBMV can be distinguished by a slightly larger molecular mass due to the addition of the CCPGCC sequence and the presence of a higher abundance of incompletely denatured dimers. That is likely due to the cysteines in the FIAsh dye-binding sequence that may become crosslinked. A small amount of dimer is typically observed in the WT BMV virion. The bottom two electron micrographs are uranyl acetate-stained samples of the WT and fBMV preparations. **C**, Ability of fBMV to enter barley protoplasts. Confocal microscopy images of barley protoplasts treated with the FIAsh dye or with fBMV made fluorescent by binding the FIAsh dye. Barley protoplasts were imaged 5 min after the addition of the fBMV or mock addition of the BMV buffer. Panels a and b were taken with a filter for the FIAsh dye, panels c and d were bright field images of the same cells. Panels e and f contain merged images. fBMV residues are clearly visualized within the protoplasts, and furthermore, several viruses are visualized within the same protoplast.

Given that the fBMV virions purified in the same manner as the WT and had a similar morphology, we added FIAsh dye to the fBMV, to render it fluorescent, and examined it for entry into barley protoplasts. We note that FIAsh is minimally fluorescent until it is coordinated by the tetracysteine motif in the CCPGCC sequence (Hoffmann et al. 2005). The images in Figure 1C were collected 5 min after the addition of fBMV to protoplasts. Fluorescence signals were detected in the sample treated with fBMV but not in samples with only the FIAsh dye or unlabeled BMV (Fig. 1C and data not shown). Superimposition of the fluorescent signal with the bright-field images shows that fBMV was inside the outer membrane of the protoplasts. This assay does not inform us about the release of the BMV RNAs, and additional tracking of BMV over time should be the subject of a different study. However, the results confirm the Western blot result (Fig. 1A) that BMV can rapidly enter protoplasts and that several virus particles can enter the same protoplast.

Recombinant CaP–green fluorescent protein (GFP) fusions.

To better manipulate the BMV CaP for entry assays, we produced the recombinant BMV CaP fused to GFP in a protein named CaP-G (Fig. 2A). Recombinant CaP-G was soluble and was fluorescent after purification (Fig. 2B). When added to the BMV protoplasts, CaP-G was found within protoplasts as punctate spots minutes after their addition to protoplasts (Fig. 2C, panels a to d). GFP alone did not exhibit fluorescence inside the cells (Fig. 2D, panels m to p). These results indicate that the recombinant BMV CaP has the ability to enter plant protoplasts.

We hypothesize that entry into protoplasts requires the ARM that resides at positions 9 to 22 of the BMV CaP. The N-terminal 22 residues of the CaP were fused to GFP to result in protein CPNT-G. When incubated with barley protoplasts, CPNT-G exhibited the same punctate distribution as CaP-G (Fig. 2C, panels e to h). A control named V-G, which contains a randomly selected sequence (MGRDPNSSSVVDKL) fused to GFP, did not exhibit fluorescence inside cells (Fig. 2C, panels i to l). These results confirmed that the N-terminal arginine-rich sequence of the BMV CaP could act as a CPP.

While the BMV CaP could enter protoplasts, we wanted to address whether the plant cell wall would affect the entry of CPNT-G or CaP-G. When added to *Arabidopsis* seedlings, CPNT-G was easily detected within cells in the root hairs (Fig. 2D, panels a to d), with a diffuse distribution as well as an accumulation of signal in membranes in root epidermal cells within 5 min (Fig. 2D, inset, panel a). Similar results were observed in barley roots (data not shown). Thus, it seems CPNT-G can enter some intact plant tissues. However, no significant signal was detected in hypocotyls when introduced topically (Fig. 2D, panel a), suggesting that tissue-specific barriers to cellular entry exist. CPNT-G was also found to enter the mesophyll cells of barley leaves (Fig. 2D, panel e). In contrast, topically applied CaP-G accumulated poorly in the root hairs and in mesophyll cells and its fluorescence was predominantly trapped within the interstitial areas between mesophyll cells (Fig. 2D, panel f). These results indicate that the cell wall, the extracellular material, or both can prevent the entry of the majority of the intact BMV CaP. However, the entire virion, the full-length CaP, and the arginine-rich N-terminal tail of the CaP are all able to cross the plasma membrane of plant cells.

CPNT is a CPP.

The ability of CPNT-G to enter plant cells suggests that CPP derived from the BMV CaP could be useful in biotechnology applications, we seek to determine whether CPNT was sufficient to traverse cellular membranes. CPNT was chemically

synthesized to contain six C-terminal residues to bind the dye FIAsh. We also tested a peptide consisting of residues 9 to 22 of the CaP N-terminal tail (named CP9-22) as well as the comparable sequence in which the six arginines were replaced with serines (CP9-22S). We also had synthesized residues 12 to 22 with five arginines (CP12-22), as well as residues 23 to 34 (CP23-34), which lacks an obvious amphipathic helix motif (Fig. 3A). After labeling with FIAsh dye, the peptides were ectopically added to *Arabidopsis* seedlings. Peptides CP9-22, CP12-22, and CPNT were found to enter the root cells, and the FIAsh signal accumulated on the membrane in root hair and also the root tip (Figs. 3B, panels d to l and 4A). No signal was detected in roots treated with CP9-22S or CP23-34 (Fig. 3A and B, panels a to c and m to o; Supplementary Table S1). CPNT and CP9-22 were also observed to accumulate mostly in the membranes of barley root-hair cells, while CP9-22S and CP23-34 did not (Fig. 3C and data not shown). These results demonstrate that the N-terminal portion of the BMV CaP can act as a CPP in *Arabidopsis* and barley roots.

Next, we examined whether CPNT can enter mesophyll cells from barley leaves. CPNT was internalized, resulting in a diffuse distribution inside the cell (Fig. 3D). CP9-22S and other peptides that lack an ARM did not appear to enter cells (Fig. 3D). A higher magnification image of CPNT at 60 min after introduction showed that some CPNT was localized in punctate spots (Fig. 3E). These results show that peptides derived from the N-terminus of the BMV CaP can permeate both the barley mesophyll cell wall and cell plasma membrane. Treatment of *Arabidopsis* seedlings with 5 to 20 μ M of CPNT or CPNT-G for 2 h had no obvious effects on the survival of *Arabidopsis* seedlings, suggesting that the CPP are not obviously toxic to seedlings (Supplementary Table S2).

Effects of inhibitors on CPNT uptake.

Chugh and associates (2009) reported that endocytic and macropinocytosis inhibitors did not significantly affect the internalization of the Tat peptide into plant cells. However, the ability of the Tat CPP to deliver GFP and glucuronidase enzyme was reduced by a macropinocytosis inhibitor in plant cells (Chang et al. 2007; Chugh et al. 2009). To examine the mechanism of CPNT internalization, several inhibitors were used in *Arabidopsis* root cells, in which they accumulated in the membranes of the meristem region of the root tip (Fig. 4A, panels a to c). Three inhibitors, oryzalin (a microtubule polymerization inhibitor), LatB (an inhibitor of actin polymerization), and EIPA (5-(N-ethyl-N-isopropyl)-amiloride, an inhibitor of Na^+/H^+ exchange-specific macropinocytosis), affected the cell cycle and induced the condensed chromosomes (Walker et al. 2007) (Fig. 4A, panels a to e, h, and k). However, only the EIPA reduced the localization of CPNT. Quantification of the signal revealed that the reduction was by 80% in three independently treated sets of samples, and the same effects were observed with either 100 or 200 μ M of EIPA (Fig. 4A, panels d to f, and B; Supplementary Fig. S1). Treatment with LatB, oryzalin, or the exocytosis inhibitor Brefeldin A (BFA) caused only slight reduction in the fluorescence of the CPNT peptide but did not alter the cellular distribution of CPNT (Fig. 4A and B). These results suggest that macropinocytosis is involved in the CPNT entry.

Nickel, which inhibits the membrane H^+ -ATPase activity (Janicka-Russak et al. 2008), did not affect CPNT entry into cells but dramatically changed the localization of CPNT from its normal accumulation at the periphery of cells to punctate distribution. ZnCl_2 did not have this effect at comparable concentrations tested (Fig. 4A, compare panels p to u). These results suggest that membrane H^+ -ATPase affected the intracellular distribution of the CPNT after its entry into cells.

CPNT can mediate GFP uptake in trans.

We wanted to examine whether CPNT could increase uptake of foreign proteins. CPNT was mixed with GFP at a 10 to 1 molar ratio and was added to mesophyll cells of barley and *N.*

benthamiana (Fig. 5A, panels d to f and j to l). In both cell types, the presence of CPNT was associated with GFP being accumulated in mesophyll cell membranes, while CP9-22S did not have this effect (Fig. 5A, panels a to c and g to i). In both

Fig. 2. *Brome mosaic virus* (BMV) capsid protein (CaP) entering into plant tissues. **A**, Schematics of the green fluorescent protein (GFP)-containing fusion proteins used in this assay. CaP-G, CPNT-G, and V-G contain, respectively, the full-length BMV CaP of 189 residues, the N-terminal residues 1 to 22 of the CaP, and a control sequence (GRDPNSSVDKL) fused to the GFP cDNA sequence. Within the names of the construct, the letter G denotes GFP. The green box denotes the sequence coding for GFP, and the black box represents the six-histidine tag added to the C-terminus of the GFP-coding sequence. **B**, The top panel shows a Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis image of the purified GFP fusion protein used in the entry assays. All proteins were expressed in recombinant *Escherichia coli*, and were purified by affinity chromatography. The protein labeled “Contr.” was not used in the analysis. The molecular-weight markers were from Invitrogen Inc. (San Diego, CA, U.S.A.) The bottom panel shows that the various purified proteins are fluorescent in solution. **C**, Confocal microscopy images of the fusion proteins in barley protoplasts. The protoplasts were treated with 3 μ M of the recombinant proteins for 30 min. GS = GFP signal, CS = chlorophyll, BF = bright field, OL = overlay of all of the images. The scale bar denotes 10 μ m. The identities of the proteins used for the treatments are shown to the left of the images. **D**, Entry of the recombinant proteins into *Arabidopsis* root and barley leaf mesophyll cells. Three-day-old *Arabidopsis* and 5-day-old barley leaf mesophyll cells were treated for 1 h with 3 μ M of the proteins shown to the left of the micrograph. The GFP signal is colored green and propidium iodide red. Whether samples were from *Arabidopsis* or barley is noted above the micrographs. The scale bar is 250 μ m for panels a through d, and 75 μ m for panels e to h. The hypocotyl in the *Arabidopsis* seedling is to the right of the green root hairs and flanked by the two insets.

barley and *N. benthamiana* leaf mesophyll cells, GFP primarily accumulated in tonoplast membranes (Fig. 5A, panel d, and B). Preferential accumulation in the corners of cell was also observed in *N. benthamiana* (Fig. 5B, panels d to f). Taken together, the results show that CPNT could act in trans to deliver GFP protein into cells.

We have also attempted to determine whether CPP present in trans of the BMV virion would affect the kinetics of virion entry into protoplasts. CPNT or CP9-22S present at 25 molar excess of the virion had only a modest effect on the detection of the BMV CaP present within the protoplasts (Supplementary Fig. S2A). The N-terminal tails in the CaP subunits of the

BMV virions may prevent us from observing any additional effects of the CPNT peptide. It is not currently possible to produce BMV virion in vitro or in vivo without the N-terminal tail (Sacher and Ahlquist 1989). The entry of the virions into protoplasts was reproducibly decreased in the presence of EIPA. However, the effect was modest when compared with that on the CPNT peptide.

CPNT can deliver BMV RNAs and double-stranded (ds)RNA into protoplasts.

We seek to determine whether CPNT can deliver BMV RNAs into plant cells. Addition of BMV RNAs in the absence

Fig. 3. The CPNT peptide can penetrate cells. **A**, Sequences of the peptides used in this study. All peptides were chemically synthesized to include a C-terminal FIAsh dye-binding sequence CCPGCC (blue letters). The arginines in CP9-22 that were substituted to serines in CP9-22S are underlined. **B**, Entry of peptides into *Arabidopsis* root. Three-day-old seedlings were treated with 5 μ M of FIAsh-labeled peptide solution for 1 h. The peptides used are labeled to the left of the images. Each treatment used a minimum of five seedlings and with consistent results. The scale bar denotes 250 μ m. **C**, CPNT entry into barley root cells. Three-day-old seedlings were treated with CP9-22S (panels a to c) or CPNT peptide (panels d to f) for 1 h prior to the collection of the images by microscopy. The scale bar denotes 250 μ m. PI denotes staining by propidium iodide. **D**, CPNT entry in barley leaf mesophyll cells. Leaves of 5- to 6-day-old seedlings were incubated with CP9-22S (a to c) or the CPNT peptide (d to f) for 1 h. The scale bar denotes 20 μ m. **E**, High resolution of CPNT distribution in barley leaf mesophyll cells. The scale bar denotes 10 μ m.

of CNPT to barley roots yielded no Western blot signals for the CaP after two days, due to either a lack of delivery or a lack of BMV gene expression in roots. Therefore, we generated protoplasts from barley leaves, a standard assay system for BMV replication (Hema and Kao 2004; Kroner et al. 1990). The BMV genomic RNAs and either CPNT or CP9-22S were mixed at three molar ratios, and the mixtures were added to barley protoplasts. After a 24-h incubation, the cells were processed for Northern blot analysis and were probed to detect the common 3' untranslated region of the BMV RNAs. BMV RNA was detected in protoplasts treated with the CPNT peptide, while no RNA signal was detected in protoplasts

treated with CP9-22S (Fig. 6A). We note that the subgenomic BMV RNA4 was observed despite being absent in the inoculum, thus demonstrating BMV RNA gene expression. We also confirmed the presence of the CaP in samples treated with CPNT, but CaP was absent in the samples treated with CP9-22S (Fig. 6B). These results show that the CPNT could deliver BMV RNAs into plant cells and that the RNAs could undergo the process expected of BMV infection.

A potential application with the CPP is to deliver dsRNAs that could act as silencing RNAs. The well-characterized phytoene desaturase (*PDS*) gene was used as a silencing target (Holzberg et al. 2002). Two complementary strands of RNA of

Fig. 4. Effects of various inhibitors on CPNT internalization. **A**, CPNT internalization in *Arabidopsis* root cells. Two-day-old seedlings were treated for 1 h with the inhibitors shown to the left of the images. The tissues were then incubated with FlAsH-labeled CPNT (20 μ M) for 1 h. NA (panels a to c) denotes samples not treated with inhibitor. The inhibitors and the final concentration used in the treatments are: 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), 200 μ M (panels d to f); LatB, 80 μ M (panels g to i); oryzalin, 200 μ M (panels j to l); Brefeldin A (BFA), 200 μ M (panels m to o); NiCl₂, 200 μ M (panels p to r); and ZnCl₂, 200 μ M (panels s to u). The scale bars denote 50 μ m. **B**, Quantification of the inhibition on CPNT entry into *Arabidopsis* root cells. NA (without inhibitor treatment) was set to 100%. FlAsH signal was quantified using Leica LAS AF Lite software. At least five seedlings were analyzed for each treatment.

the barley *PDS* gene were synthesized by in vitro transcription and were annealed in vitro to generate a dsRNA (Fig. 7A and B). The dsRNA was mixed together with a 25-fold molar excess of CPNT or CP9-22S and was then added to freshly prepared barley protoplasts. After incubation for 48 h, the *PDS* RNA level was examined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The *PDS* RNA introduced to the cell along with CPNT was reduced to 50% in comparison to CP9-22S (Fig. 7C). This experiment was consistent in six independently tested samples. Furthermore, the (+) and (–) strands of the *PDS* dsRNA did not reduce levels of RNA (Fig. 7D), which is in agreement with dsRNA being required for the silencing of plant gene expression.

CPP from other viruses.

Small icosahedral viruses often possess an N-terminal ARM in their CaP that likely coordinates RNA packaging and may even regulate cellular innate immune responses (Belyi and Muthukumar 2006; Qu and Morris 1997). We explored whether other plant viruses have sequences that could act as CPP. The *Barley yellow dwarf virus* CaP was selected as a potential CPP, along with sequences from two geminiviruses that contain single-stranded (ss)DNA genomes (Fig. 8A). All three sequences were synthesized to contain the tetracycline sequence to bind the FIASH dye and the fluorescent peptide used to treat

Arabidopsis and barley roots and barley leaf mesophyll cells (Fig. 8B). FIASH signal was detected in samples treated with all three peptides but not in samples treated with CP23-34, which lacks an ARM (Fig. 8B).

DISCUSSION

This study attempted to address the roles of the viral capsid in the entry of cells as well as CPP derived from a plant viral CaP. We demonstrate that the intact BMV virion and recombinant BMV CaP fused with GFP can enter the membranes of protoplasts. In plant tissues, the virion was mostly trapped by the cell wall, suggesting that if the CaP has a role of protein transduction in BMV entry, it must follow another mechanism to breach the interstitial and cell-wall materials. This result is consistent with the reported observation that the plant cell wall has the ability to exclude materials larger in mass than 40 kDa (Schwuchow et al. 2002).

We also addressed potential uses for the CPP derived from plant viral CaP. The N-terminal tail containing the ARM is required to penetrate cells. However, the intracellular location of the BMV-derived CPP depends on the cell type. In root cells from *Arabidopsis* and barley, the BMV CPP primarily accumulated in the plasma membrane. In leaf mesophyll cells, the CPP had a diffuse localization in the cells, suggestive of mem-

Fig. 5. CPNT can deliver green fluorescent protein (GFP) into mesophyll cells. **A**, Results from barley and *Nicotiana benthamiana* leaf mesophyll cells. The cells were treated for 1 h with 5 μ M of the V-G protein along with a 10-molar excess of the CP9-22S (panels a to c and g to i) or the CPNT (panels d to f and j to l). The scale bar denotes 50 μ m for a to f and 75 μ m for g to i. **B**, CPNT-GFP fusion protein entry into *N. benthamiana* leaf mesophyll cells. *N. benthamiana* leaf mesophyll cells were treated with CPNT-GFP (3 μ M) and V-GFP (3 μ M) for 1 h. The scale bar denotes 250 μ m for a to c and g to i and 50 μ m for d to f. **C**, A higher magnification image of the barley and *N. benthamiana* mesophyll cells that had taken up the fluorescent signal.

branes such as the tonoplast (Fig. 3). The location of CPNT in cellular membranes is a feature shared with the cell-penetrating peptides from *Flock house virus* (Nakase et al. 2009).

The localization of the BMV-derived CPP depends on the concentration of the peptides. In *Arabidopsis*, the CPNT can be detected easily with concentrations as low as 5 μ M in root hairs (Fig. 3), while approximately 20 μ M of peptide is needed for detection in the root tip (Fig. 4). These data all suggest that the physiology of the cells, likely the materials in the extracellular matrix, will be an important determinant in the efficiency of CPP entry.

Macropinocytosis and actin organization are involved in the internalization of Tat, arginine-rich peptide, and FHV peptide in mammalian cells (Nakase et al. 2007, 2009; Wadia et al. 2004). We observed that the macropinocytosis inhibitor EIPA decreased CPNT entry in plant root cells (Fig. 4A, panel d). However, inhibitors of actin and microtubule polymerization, LatB or oryzalin, did not affect the internalization of CPNT in *Arabidopsis* root cells, even though these inhibitors affect the accumulation of the condensed chromosomes in the treated cells (Fig. 4A, panels d to l). The endocytosis inhibitor BFA did not inhibit CPNT entry (Fig. 4A, panels m to o). This suggests CPNT entry is mainly through the macropinocytosis pathway and is independent of the endosome- or actin-mediated process. Jiao and associates (2009) also showed localization by the endocytosis pathway, and direct translocation depended strongly on the concentration of the cell-penetrating peptide.

Nickel but not zinc changed the membrane localization of CPNT to punctate distribution (Fig. 4A, panels p to u). Nickel and zinc are stored in vacuoles, and their uptake uses plasma membrane-associated proton pumps and vacuolar H⁺-ATPases (Krebs et al. 2010; Nishimura et al. 1998). The concentration of nickel we used has been reported to inhibit the H⁺-ATPase activity (Janicka-Russak et al. 2008), a process that may be related to macropinocytosis requiring proper Na⁺ and H⁺ exchange (Dettmer et al. 2006; Janicka-Russak et al. 2008; Kerr and Teasdale 2009; Mercer and Helenius 2008). The effect of nickel suggests that the proton gradient in the cell is also critical for the cellular localization of CPNT.

The BMV CPNT could be useful in delivering protein or RNA to plants. We demonstrated that CPNT can facilitate GFP entry into mesophyll cells and viral or dsRNA into barley protoplasts (Figs. 5 to 7). The efficiency of GFP delivery was higher when CPNT was fused to GFP than when CPNT was present in trans. However, the cellular localization in leaf mesophyll cells appears to be similar in both of the two methods (Fig. 5). This is consistent with reports on Tat peptide that can deliver plasmid DNA into the microspores of a hybrid of wheat, triticale (Chugh et al. 2009). Others have found that the HIV Tat and Arg9 peptides could deliver GFP to onion nuclei (Chang et al. 2007; Chugh and Eudes 2007), but we did not observe nuclear localization of CPNT or CPNT-GFP in our system. The ability of CPNT to deliver dsRNA into cells and induce gene silencing could be taken advantage of for biotechnology applications. We note that CPNT did not mediate silencing with ssRNAs, possibly due to either a lack of delivery or that ssRNAs can not function in silencing.

Finally, it is interesting to note that CPP are present in viruses that can infect many different hosts, from human to insects to plants. The portion of the BMV capsid that can be made into active CPP is also the portion of the capsid that interacts with the viral RNA during encapsidation (Duggal and Hall 1993; Hema et al. 2010; Sacher and Ahlquist 1989). The Tat CPP derived from HIV is also involved in contacting RNA (Frankel and Pabo 1988), further suggesting a link between interaction with nucleic acids and cell membranes for at least some classes of CPP.

MATERIALS AND METHODS

Materials.

Arabidopsis thaliana (Col-0) seedlings were grown on Murashige-Skoog medium for 2 to 3 days, with cycles of 16 h of light and 8 h of dark, at 22°C. Barley (*Hordeum vulgare*) seedlings germinated for 3 days in sterile water were used for CPP uptake experiments, and sections of leaves from plants grown in soil for 5 to 6 days were placed on microscope slides for treatment with virions or peptides. Barley protoplasts were iso-

Fig. 6. CPNT can deliver *Brome mosaic virus* (BMV) RNA into protoplasts. **A**, Northern blot analysis of the BMV RNAs that were introduced into barley protoplasts (input) in the presence of CPNT or CP9-22S. The blot was probed with a riboprobe to detect the 3' untranslated sequence present on positive-strand BMV RNAs. The bottom gel image shows the ethidium bromide-stained total RNAs present in protoplasts prior to use in the Northern blot assay. Only BMV RNA1, RNA2, and RNA3 were introduced into the protoplasts; hence, the production of BMV RNA4 demonstrates that RNA replication and transcription has taken place. All of the samples were from one Northern blot, but the controls are spliced to allow proper visualization of the relevant bands. cRNA denotes cellular RNA used as a loading control. **B**, Western blot that detects the BMV CP. The protoplast samples were processed in parallel to the ones used for Northern blot analysis in panel A. The L.C. (loading control) was a protein band from the Western blot that has been stained with Ponceau Red.

lated from 6-day-old barley as previously described (Kroner et al. 1990). We note that additions of virions, proteins, or CPP in transduction experiments did not use PEG or Ca^{2+} to mediate entry. Four-week-old *N. benthamiana* seedlings were used as the source for leaf mesophyll cells and to prepare BMV virions. The virions were generated by infiltration with recombinant *Agrobacterium tumefaciens* that can recapitulate the BMV infection for 7 days, as described by Gopinath and associates (2005). Viruses were extracted by the protocol in Yi and associates (2009).

Construction of a fluorescent *Brome mosaic virus*.

Agrobacterium construct expressing BMV RNA3 was used in a site-directed mutagenesis to insert the 18 nucleotides encoding the FIAsh dye-binding sequence of CCPGCC. The insertion was selected to be between residues 23 and 24, since this location did not affect the arginine-rich sequence and was less likely to affect the globular domains that mediate capsid subunit interactions. The mutagenesis was as described in Yi and Kao (2008), and primers fBMV_F and fBMV_R were used to amplify (Supplementary Table S3). The construct was

Fig. 7. CPNT can deliver double stranded (ds)RNA into protoplasts. **A**, Schematic of the barley phytoene desaturase (*PDS*) gene, the sequence used to generate the dsRNAs for use in knockdown experiments, and the locations of the primers (marked by arrows) used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The sequence of the gene is from the National Center for Biotechnology Information (AY062039). **B**, The positive- and negative-strand RNAs produced by in vitro transcription, used to silence the *PDS* gene. The dsRNA was generated by annealing the single-stranded RNAs at 95°C for 5 min and slowly cooling the sample over a 1-h period to room temperature. The two single-stranded RNAs are labeled with (-) or (+), and the annealed RNA is labeled ds. **C**, Results from qRT-PCR of *PDS* gene expression. Protoplasts were treated with 2 μM of CPNT (black columns) or CP9-22S (gray columns) mixed together with the annealed RNA (1 μg). The protoplasts were incubated for 24 and 48 h, respectively, prior to harvesting of the total RNA for analysis. **D**, qRT-PCR results of *PDS* gene expression in protoplasts treated with 2 μM of CPNT or CP9-22S with or without single-stranded RNA (1 μg). The data is from four independent replicates for each treatment. Signal from the actin gene was used as an internal control.

made in *Escherichia coli* and was transferred to *A. tumefaciens* as described in Gopinath and associates (2005). An equal mixture of the *Agrobacterium* cultures that can express BMV RNA1, RNA2, and the FIAsh-tagged RNA3 were infiltrated into *N. benthamiana* for 8 days, and the virions were purified using sucrose and CsCl density gradients as described in Yi and Kao (2008).

Peptide synthesis, cell entry, and FIAsh dye labeling.

Peptides used in this study were synthesized by Peptide 2.0 Inc. (Chantilly, VA, U.S.A.) and contained a C-terminal FIAsh dye-binding sequence (CCPGCC). Labeling with the FIAsh dye used a 1:1 mix of 5 μ M peptide and FIAsh (Invitrogen Inc., San Diego, CA, U.S.A.) that was incubated on ice for 30 min. *Arabidopsis* and barley seedlings were treated for up to 1 h in this mixed solution, followed by washing three times with 0.05% SDS, before mounting on a slide for imaging.

Fluorescence images were obtained using a Leica SP5 scanning confocal microscope. To detect the fluorescence of GFP and the FIAsh dye, the excitation wavelength was 488 nm and the emission filter was set between 505 and 530 nm. To detect propidium iodide and chlorophyll, the emission wavelength was between 640 to 710 nm. Quantification of the signals from micrographs used the Leica LAS AF Lite software.

Construction of GFP fusion proteins, expression, purification, and cell-entry experiments.

The cDNA coding for GFP was amplified from the PBSSK-GFP plasmid with primers GFP_For and GFP_Rev, followed by digestion with *Hind*III and *Xho*I and cloning into pET-21b to generate plasmid pET-21b-V-GFP for V-GFP protein expression. The forward primer CP_For was used with CPNT_Rev or CP_Rev to amplify the full-length CaP or CPNT, respectively. The cDNAs were cloned into plasmid pET-21b-V-GFP vector to result in constructs CPNT-G and CaP-G. For GFP-alone expression construction, primers GFP_For and GFP_Rev were used and digestion with *Nhe*I and *Xho*I, further cloned into pET-21b.

E. coli BL21(star) was used to express the recombinant fusion proteins. The cultures were grown to an optical density at 600 nm of 0.5, at which time isopropyl- β -D-thiogalactoside was added to a final concentration of 0.5 mM, to induce recombinant protein expression. After a 12-h induction at 18°C, the cells were harvested by centrifugation and were resuspended in lysis buffer (50 mM Tris, 300 mM NaCl, 1 mM β -mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 20 mM imidazole, pH 7.8). The cells were lysed by sonification, and the soluble material was clarified with a 10,000 $\times$ g centrifugation prior to capturing of the His-tagged proteins by use of a His-resin from Clontech (Mountain View, CA, U.S.A.). After extensive washed with the lysis buffer, proteins were eluted in the lysis buffer amended with 470 mM imidazole and concentrated with buffer washes to remove imidazole. Protein concentrations were determined by the Bradford protein assay using BSA as a standard (Sigma-Aldrich Inc. St. Louis).

Cell-entry experiments performed with intact seedlings or leaves were treated with 3 μ M of purified protein. Protoplasts were incubated for 30 min prior to processing for confocal microscopy.

Inhibitor treatments.

EIPA, BFA, and oryzalin (Calbiochem, Gibbstown, NJ, U.S.A.) were dissolved in dimethylsulfoxide (DMSO) in 10-mM stock solutions. Latrunculin B (LatB) was made as a 4-mM stock solution in DMSO. Final concentration of EIPA, BFA, and oryzalin were 200 or 100 μ M, while LatB was at 80 or 40 μ M. Final concentrations of NiCl_2 or ZnCl_2 were 200 or 100 μ M. Mock-treated samples were subjected to DMSO at the same concentration as treatment with the various inhibitors.

RNA production, delivery, and extraction from protoplasts.

BMV RNA1, RNA2, and RNA3 were transcribed in vitro with the AmpliCap-Max T7 kit from linearized plasmid (Janda et al. 1987) (Epicentre Technologies, Madison, WI, U.S.A.). The barley *PDS* gene was designed as the target gene for RNA inter-

Fig. 8. Cell-penetrating peptides (CPP) from three other plant viruses. **A**, Sequences from the capsid protein (CaP) of plant viruses tested for function as CPP. The sequences were identified by visual surveys of over three dozen plant viral capsid sequences. The numbers in the peptides denote the residues in the CaP from the viruses from which the sequences were derived. BYDV = Barley yellow dwarf virus; TCSV = Tobacco curly shoot virus; BeYDV = Bean yellow dwarf virus. CP23-34 is the Brome mosaic virus peptide that lacks an arginine-rich motif. The FIAsh dye-binding sequence is shown in blue. **B**, Confocal micrographs of *Arabidopsis* roots and barley mesophyll cells treated with FIAsh-labeled peptides. The scale bar is 250 μ m for a through d and 75 μ m for e through h. The results are consistent for treatments between 5 and 20 μ M of the four peptides.

fering (Holzberg et al. 2002). The two strands of the dsRNAs were made separately by in vitro transcription. Transcription of the (+)-strand RNA used oligonucleotides T7_bPDS_For and T7_bPDS_For_RC. Transcription of (–)-strand RNA used DNA oligos T7_bPDS_Rev and T7_bPDS_Rev_RC. The two transcripts were purified by gel-filtration chromatography and were then mixed in equal molar ratios and heated to 95°C for 5 min, followed by removal of the heating block to the bench top to allow slow cooling of the samples for 1 h.

Transfection of barley protoplasts used an equal molar ratio of the three BMV genomic RNAs mixed with CPNT or CP9-22S peptides. dsRNA was mixed with CPNT or CP9-22S peptides on ice for 30 min in buffer P (0.2 mM KH₂PO₄, 1 mM KNO₃, 1 mM MgSO₄, 1 μM KI, 0.1 μM CuSO₄, 10 mM CaCl₂, 10% mannitol, pH 6.5) before adding to protoplasts. After the indicated time of treatment, the protoplasts were washed three times, before adding RNA extraction buffer (0.1 M glycine, 40 mM EDTA, 100 mM NaCl, and 2% SDS). The sample was subjected to extraction with an equal volume of phenol and chloroform (1:1 ratio) prior to precipitating the total RNAs with an equal volume of isopropanol.

qRT-PCR was performed with 2 μg of total RNA. RT used the M-MLV reverse transcriptase (Ambion, Austin, TX, U.S.A.), while the PCR used the iQ SYBR Green supermix kit (Bio-Rad, Hercules, CA, U.S.A.). Primers bPDS_For and bPDS_Rev were used for barley *PDS* gene expression detection. Primers bActin_For and bActin_Rev were used for actin gene-expression detection.

Northern and Western blots and RT-PCR.

RNAs used for Northern blots were denatured with glyoxal for 1 h and were electrophoresed on 1.2% agarose gels as described by Sivakumaran and associates (2003). The probe used detected the 3′ untranslated region of the BMV RNAs, a region nearly identical in the genomic and subgenomic BMV RNAs. Western blots used protoplasts that were dissolved in the Laemmli SDS-PAGE buffer and were separated on 4 to 12% Bis-Tris gels. After gel electrophoresis, proteins were transferred to polyvinylidene difluoride membrane for Western blot detection with rabbit anti-CaP antibody. The secondary antibody was goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase. The signal was visualized by use of the ECL Plus kit (Amersham, Arlington, IL, U.S.A.). The protocol for RT-PCR analysis of BMV RNAs was previously described by Hema and associates (2010).

CPP from other plant viruses.

CaP residues 13 to 25 of *Tobacco curly shoot virus* (ACZ71224, PASKVRRRLNFDS) and residues 8 to 16 of *Bean yellow dwarf virus* (ABE67101, KRKRSGGDS) were selected as potential CPP sequences from the ssDNA *Begomovirus* and *Mastrevirus* geminiviruses, respectively. A peptide containing CaP residues 15 to 26 from the *Barley yellow dwarf virus* (NP_620067, PRRARRVSAVRR) was used as another potential CPP from a positive-sense ssRNA virus in addition to BMV. All three peptides were synthesized with a C-terminal FIASH dye-binding sequence and were labeled with FIASH prior to use in cell-entry experiments. *Arabidopsis* seedlings and barley leaf mesophyll cells were treated with from 5 to 20 μM FIASH-labeled peptides for 1 h prior to examination of the samples by confocal microscopy.

ACKNOWLEDGMENTS

We thank L. Kao for critically reading the manuscript and S. Shaw and K. Fujisaki for many helpful discussions on manipulation of plant tissues and cells. We thank our lab members P. Ni, H. Masarapu, and E. McKin-

ney for technical assistance and plant care and P. Ni for the electron microscopy images. The IUB Light Microscopy Imaging Center provided microscopy resources and we thank J. Powers for his guidance.

This work was supported by the National Science Foundation grant MCB0641362 and NIH grant 1R01AI090280 from the NIAID.

LITERATURE CITED

- Annamalai, P., Apte, S., Wilkens, S. and Rao, A. L. N. 2005. Deletion of highly conserved arginine-rich RNA binding motif in *Cowpea chlorotic mottle virus* capsid protein results in virion structural alterations and RNA packaging constraints. *J. Virol.* 79:3277-3288.
- Belyi, V. A. and Muthukumar, M. 2006. Electrostatic origin of the genome packing in viruses. *Proc. Natl. Acad. Sci. U.S.A.* 103:17174-17178.
- Chang, M., Chou, J.-C., Chen, C.-P., Liu, B. R. and Lee, H.-J. 2007. Non-covalent protein transduction in plant cells by macropinocytosis. *New Phytol.* 174:46-56.
- Chugh, A., Amundsen, E., and Eudes, F. 2009. Translocation of cell-penetrating peptides and delivery of their cargoes in triticales microspores. *Plant Cell Rep.* 28:801-810.
- Chugh, A., and Eudes, F. 2007. Translocation and nuclear accumulation of monomer and dimer of HIV-1 Tat basic domain in triticales mesophyll protoplasts. *BBA-Biomembranes* 1768:419-426.
- Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. 1994. The third helix of the Antennapedia homeodomain translocates through biological membranes. *J. Biol. Chem.* 269:10444-10450.
- Dettmer, J., Hong-Hermesdorf, A., Stierhof, Y.-D., and Schumacher, K. 2006. Vacuolar H⁺-ATPase activity is required for endocytic and secretory trafficking in *Arabidopsis*. *Plant Cell* 18:715-730.
- Drin, G., Cottin, S., Blanc, E., Rees, A. R., and Temsamani, J. 2003. Studies on the internalization mechanism of cationic cell-penetrating peptides. *J. Biol. Chem.* 278:31192-31201.
- Duggal, R., and Hall, T. C. 1993. Identification of domains in *Brome mosaic virus* RNA-1 and coat protein necessary for specific interaction and encapsidation. *J. Virol.* 67:6406-6412.
- Elmqvist, A., Lindgren, M., Bartfai, T., and Langel, Ü. 2001. VE-cadherin-derived cell-penetrating peptide, pVEC, with carrier functions. *Exp. Cell Res.* 269:237-244.
- Fernandez-Carnead, J., Kogan, M. J., Pujals, S., and Giralt, E. 2004. Amphipathic peptides and drug delivery. *Biopolymers* 76:196-203.
- Ford, K. G., Souberbielle, B. E., Darling, D., and Farzaneh, F. 2001. Protein transduction: An alternative to genetic intervention? *Gene Ther.* 8:1-4.
- Frankel, A. D., and Pabo, C. O. 1988. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* 55:1189-1193.
- Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugiyama, Y. 2001. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J. Biol. Chem.* 276:5836-5840.
- Gerbail-Chaloin, S., Gondeau, C., Aldrian-Herrada, G., Heitz, F., Gauthier-Rouvière, C., and Divita, G. 2007. First step of the cell-penetrating peptide mechanism involves Rac1 GTPase-dependent actin-network remodelling. *Biol. Cell* 99:223-238.
- Gopinath, K., Dragnea, B., and Kao, C. 2005. Interaction between *Brome mosaic virus* proteins and RNAs: Effects on RNA replication, protein expression, and RNA stability. *J. Virol.* 79:14222-14234.
- Green, M., and Loewenstein, P. M. 1988. Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. *Cell* 55:1179-1188.
- Hema, M., and Kao, C. C. 2004. Template sequence near the initiation nucleotide can modulate *Brome mosaic virus* RNA accumulation in plant protoplasts. *J. Virol.* 78:1169-1180.
- Hema, M., Murali, A., Fujisaki, K., Tsyetkova, I., Ni, P., Vaughan, R. C., Dragnea, B., and Kao, C. C. 2010. Effects of amino acid substitutions in the *Brome mosaic virus* capsid protein on RNA encapsidation. *Mol. Plant-Microbe Interact.* 11:1433-1447.
- Hoffmann, C., Gaietta, G., Bunemann, M., Adams, S. R., Oberdorff-Maass, S., Behr, B., Vilardaga, J.-P., Tsien, R. Y., Ellisman, M. H., and Lohse, M. J. 2005. A FIASH-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nat. Meth.* 2:171-176.
- Holzberg, S., Brosio, P., Gross, C., and Pogue, G. P. 2002. *Barley stripe mosaic virus*-induced gene silencing in a monocot plant. *Plant J.* 30:315-327.
- Janda, M., and Ahlquist, P. 1993. RNA-dependent replication, transcription, and persistence of brome mosaic virus RNA replicons in *S. cerevisiae*. *Cell* 72:961-970.
- Janda, M., French, R., and Ahlquist, P. 1987. High efficiency T7 polymerase synthesis of infectious rna from cloned brome mosaic virus cDNA and effects of 5′ extensions on transcript infectivity. *Virology* 158:259-262.

- Janicka-Russak, M., Kabala, K., Burzynski, M., and Klobus, G. 2008. Response of plasma membrane H⁺-ATPase to heavy metal stress in *Cucumis sativus* roots. *J. Exp. Bot.* 59:3721-3728.
- Jiao, C.-Y., Delaroche, D., Burlina, F., Alves, I. D., Chassaing, G., and Sagan, S. 2009. Translocation and endocytosis for cell-penetrating peptide internalization. *J. Biol. Chem.* 284:33957-33965.
- Johnson, K. N., Tang, L., Johnson, J. E., and Ball, L. A. 2004. Heterologous RNA encapsidated in Pariacoto virus-like particles forms a dodecahedral cage similar to genomic RNA in wild-type virions. *J. Virol.* 78:11371-11378.
- Jones, A. T. 2007. Macropinocytosis: Searching for an endocytic identity and role in the uptake of cell penetrating peptides. *J. Cell Mol. Med.* 11:670-684.
- Kao, C. C., and Sivakumaran, K. 2000. *Brome mosaic virus*, good for an RNA virologist's basic needs. *Mol. Plant Path.* 1:91-97.
- Kerr, M. C., and Teasdale, R. D. 2009. Defining macropinocytosis. *Traffic* 10:364-371.
- Krebs, M., Beyhl, D., Görlich, E., Al-Rasheid, K. A. S., Marten, I., Stierhof, Y.-D., Hedrich, R., and Schumacher, K. 2010. *Arabidopsis* V-ATPase activity at the tonoplast is required for efficient nutrient storage but not for sodium accumulation. *Proc. Natl. Acad. Sci. U.S.A.* 107:3251-3256.
- Kroner, P. A., Young, B. M., and Ahlquist, P. 1990. Analysis of the role of brome mosaic virus 1a protein domains in RNA replication, using linker insertion mutagenesis. *J. Virol.* 64:6110-6120.
- Liebold, L. O., Revis, J., Allen, M., Oltrogge, L., Young, M., and Douglas, T. 2005. Structure transitions in *Cowpea chlorotic mottle virus* (CCMV). *Phys. Biol.* 2:S166-S172.
- Lucas, R. W., Larson, S. B., and McPherson, A. 2002. The crystallographic structure of brome mosaic virus. *J. Mol. Biol.* 317:95-108.
- Mäe, M., Myrberg, H., Jiang, Y., Paves, H., Valkna, A., and Langel, Ü. 2005. Internalisation of cell-penetrating peptides into tobacco protoplasts. *Biochim. Biophys. Acta* 1669:101-107.
- Mano, M., Teodosio, C., Paiva, A., Simoes, S., and Pedrosa de Lima, M. C. 2005. On the mechanisms of the internalization of S4(13)-PV cell-penetrating peptide. *Biochem. J.* 390:603-612.
- Mercer, J., and Helenius, A. 2008. Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. *Science* 320:531-535.
- Nakase, I., Hirose, H., Tanaka, G., Tadokoro, A., Kobayashi, S., Takeuchi, T., and Futaki, S. 2009. Cell-surface accumulation of *Flock house virus*-derived peptide leads to efficient internalization via macropinocytosis. *Mol. Ther.* 17:1868-1876.
- Nakase, I., Tadokoro, A., Kawabata, N., Takeuchi, T., Katoh, H., Hiramoto, K., Negishi, M., Nomizu, M., Sugiura, Y., and Futaki, S. 2007. Interaction of arginine-rich peptides with membrane-associated proteoglycans is crucial for induction of actin organization and macropinocytosis. *Biochem. J.* 406:492-501.
- Nishimura, K., Igarashi, K., and Kakinuma, Y. 1998. Proton gradient-driven nickel uptake by vacuolar membrane vesicles of *Saccharomyces cerevisiae*. *J. Bacteriol.* 180:1962-1964.
- Noueiry, A. O., and Ahlquist, P. 2003. *Brome mosaic virus* RNA replication: Revealing the role of the host in RNA virus replication. *Ann. Rev. Phytopath.* 41:77-98.
- Oehlke, J., Scheller, A., Wiesner, B., Krause, E., Beyermann, M., Klauschen, E., Melzig, M., and Bienert, M. 1998. Cellular uptake of an α -helical amphipathic model peptide with the potential to deliver polar compounds into the cell interior non-endocytically. *BBA-Biomembranes* 1414:127-139.
- Pacha, R. F., and Ahlquist, P. 1991. Use of bromovirus RNA3 hybrids to study template specificity in viral RNA amplification. *J. Virol.* 65:3693-3703.
- Pooga, M., Hällbrink, M., Zorko, M., and Langel, U. 1998. Cell penetration by transport. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 12:67-77.
- Qu, F., and Morris, T. 1997. Encapsidation of turnip crinkle virus is defined by a specific packaging signal and RNA size. *J. Virol.* 71:1428-1435.
- Sacher, R., and Ahlquist, P. 1989. Effects of deletions in the N-terminal basic arm of brome mosaic virus coat protein on RNA packaging and systemic infection. *J. Virol.* 63:4545-4552.
- Schwuchow, J. M., Kern, V. D., and Sack, F. D. 2002. Tip-growing cells of the moss *Ceratodon purpureus* are gravitropic in high-density media. *Plant Physiol.* 130:2095-2100.
- Sgro J.-Y., Jacrot, B., and Chroboczek, J. 1986. Identification of regions of *Brome mosaic virus* coat protein chemically cross-linked in situ to viral RNA. *Eur. J. Biochem.* 154:69-76.
- Sivakumaran, K., Hema, M., and Kao, C. C. 2003. *Brome mosaic virus* RNA syntheses in vitro and in barley protoplasts. *J. Virol.* 77:5703-5711.
- Smith, A. E., and Helenius, A. 2004. How viruses enter animal cells. *Science* 304:237-242.
- Wadia, J. S., Stan, R. V., and Dowdy, S. F. 2004. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med.* 10:310-315.
- Walker, K. L., Müller, S., Moss, D., Ehrhardt, D. W., and Smith, L. G. 2007. *Arabidopsis* TANGLED identifies the division plane throughout mitosis and cytokinesis. *Curr. Biol.* 17:1827-1836.
- Wei, T., Chen, H., Ichiki-Uehara, T., Hibino, H., and Omura, T. 2007. Entry of *Rice dwarf virus* into cultured cells of its insect vector involves clathrin-mediated endocytosis. *J. Virol.* 81:7811-7815.
- Yi, G., and Kao, C. 2008. Cis- and trans-acting functions of *Brome mosaic virus* protein 1a in genomic RNA1 replication. *J. Virol.* 82:3045-3053.
- Yi, G., Vaughan, R. C., Yarbrough, L., Dharmiah, S., and Kao, C. C. 2009. RNA binding by the *Brome mosaic virus* capsid protein and the regulation of viral RNA accumulation. *J. Mol. Biol.* 391:314-326.
- Zhou, F., Pu, Y., Wei, T., Liu, H., Deng, W., Wei, C., Ding, B., Omura, T., and Li, Y. 2007. The P2 capsid protein of the nonenveloped *rice dwarf phytoeovirus* induces membrane fusion in insect host cells. *Proc. Natl. Acad. Sci. U.S.A.* 104:19547-19552.
- Zhu, J., Gopinath, K., Murali, A., Yi, G., Hayward, S. D., Zhu, H., and Kao, C. 2007. RNA-binding proteins that inhibit RNA virus infection. *Proc. Natl. Acad. Sci. U.S.A.* 104:3129-3134.