RESEARCH PAPER

Plasma membrane-associated cation-binding protein 1-like protein negatively regulates intercellular movement of BaMV

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Abstract

To establish a successful infection, a virus needs to replicate and move cell-to-cell efficiently. We investigated whether one of the genes upregulated in *Nicotiana benthamiana* after Bamboo mosaic virus (BaMV) inoculation was involved in regulating virus movement. We revealed the gene to be a plasma membrane-associated cation-binding protein 1-like protein, designated *NbPCaP1L*. The expression of *NbPCaP1L* in *N. benthamiana* was knocked down using Tobacco rattle virus-based gene silencing and consequently the accumulation of BaMV increased significantly to that of control plants. Further analysis indicated no significant difference in the accumulation of BaMV in *NbPCaP1L* knockdown and control protoplasts, suggesting *NbPCaP1L* may affect cell-to-cell movement of BaMV. Using a viral vector expressing green fluorescent protein in the knockdown plants, the mean area of viral focus, as determined by fluorescence, was found to be larger in *NbPCaP1L* knockdown plants. Orange fluorescence protein (OFP)-fused NbPCaP1L, NbPCaP1L-OFP, was expressed in *N. benthamiana* and reduced the accumulation of BaMV to 46%. To reveal the possible interaction of viral protein with NbPCaP1L, we performed yeast two-hybrid and co-immunoprecipitation experiments. The results indicated that NbPCaP1L interacted with BaMV replicase. The results also suggested that NbPCaP1L could trap the BaMV movement RNP complex via interaction with the viral replicase in the complex and so restricted viral cell-to-cell movement.

Key words: Bamboo mosaic virus; defense protein; NbPCaP1L; *Nicotiana benthamiana*; positive-sense RNA virus; replicase; viral RNA movement.

Introduction

To complete an infection cycle, a plant virus needs to not only replicate efficiently but also move cell-to-cell successfully in the form of a virion or viral ribonucleoprotein complex (RNP) via the plasmodesmata (PD) (Hofmann *et al.*, 2007).

The latter process requires both viral-encoded movement proteins (MPs) and host factors (Scholthof, 2005; Taliansky *et al.*, 2008; Benitez-Alfonso *et al.*, 2010; Niehl and Heinlein, 2011; Schoelz *et al.*, 2011). In addition, the cytoskeleton

system is involved in transporting the viral components to the PD (Prokhnevsky *et al.*, 2005; Avisar *et al.*, 2008). Although the microtubules do not play a major role in viral RNA/protein complex trafficking in tobacco mosaic virus (TMV), it is still a key player involved in delivering the complex to microfilaments for transportation (Li *et al.*, 2011; Peña and Heinlein, 2012; Liu and Nelson, 2013). Potato mop-top virus (PMTV) possesses a triple gene block (TGB) that encodes three MPs for virus movement. Mutants with an N-terminal 84-amino acid deletion of TGB1 in PMTV, with lost activity in microtubule association, failed to show long distance movement (Agre *et al.*, 1998). A host protein NbMPB2Cb from *N. benthamiana*, a homolog of TMV-MP30 binding protein 2C, MPB2C, which is involved in organizing the cortical microtubules (Chaumont *et al.*, 2000), could regulate the movement of Potato virus X (PVX). The movement of PVX is restricted when NbMPB2Cb is overexpressed; by contrast the movement is enhanced when the expression of NbMPB2Cb is silenced (Hachez *et al.*, 2013).

Bamboo mosaic virus (BaMV) is a member of the *Potexvirus* genus of *α-Flexiviridae*. The genome of BaMV has one single-stranded positive-sense RNA approximately 6.4 kb long with a 5′ m^{7}GpppG structure and 3′ adenylates numbering ~250−300 (Lin *et al.*, 1992; Chen *et al.*, 2005). The genomic RNA of BaMV contains five open reading frames, ORFs 1 to 5. ORF 1 encodes a 155 kDa polypeptide comprising three functional domains. The N-terminal capping enzyme domain possesses S-adenosylmethionine–dependent guanylytransferase activity (Li *et al.*, 2001*a*; Huang *et al.*, 2004; Huang *et al.*, 2005); the middle part is a helicase-like domain with nucleoside triphosphatase (NTPase) and RNA 5′-triphosphatase activities (Li *et al.*, 2001*b*) and the C-terminal RNA-dependent RNA polymerase (RdRp) domain (Li *et al.*, 1998; Huang *et al.*, 2001) has viral RNA replication activity. The overlapped ORFs 2 to 4, TGBp1 to 3, encode MPs for cell-to-cell movement (Lin *et al.*, 2004; Lin *et al.*, 2006). ORF 5 encodes a 25 kDa viral capsid protein (CP) required for virion assembly, symptom development, and movement in plant cells (Lan *et al.*, 2010; Hung *et al.*, 2014*a*; Hung *et al.*, 2014*b*). Infected protoplasts and plants show two major subgenomic RNAs, 2 and 1 kb long (Tsai *et al.*, 1999; Yeh *et al.*, 1999).

Several host factors positively or negatively regulate the infection cycle of BaMV. The chloroplast phosphoglycerate kinase (Cheng *et al.*, 2013*a*), heat-shock protein 90 (Huang *et al.*, 2012), a glutathione transferase NbGSTU4 (Chen *et al.*, 2013), an exoribonuclease NbXRN4 (Lee *et al.*, 2015), and a Rab-GTPase NbRABG3f (Huang *et al.*, 2016) are involved in assisting viral RNA replication. A Ser/Thr kinase-like protein NbSTKL (Cheng *et al.*, 2013*b*) and a Rab-GTPase activation protein NbRabGAP1 (Huang *et al.*, 2013) support viral movement. By contrast, a cytoplasmic form of glyceraldehyde 3-phosphate dehydrogenase (Prasanth *et al.*, 2011) and a putative methyltransferase (Cheng *et al.*, 2009) suppress BaMV RNA replication by interacting with the 3′ UTR and replicase, respectively.

A group of proteins on the plasma membrane containing six transmembrane domains termed plasma membrane intrinsic proteins (PIPs) play roles in regulating the diffusion of water and small uncharged solutes (Agre *et al.*, 1998; Schäffner, 1998; Chaumont *et al.*, 2000; Hachez *et al.*, 2013). Some PIPs can even transport hydrogen peroxide in response to stresses (Jang *et al.*, 2012). A new class of PIPs, without any cross-membrane domain and associated with the plasma membrane via myristoylation, bind Ca^{2+}, Mg^{2+}, and Cu^{2+} and are called plasma membrane-associated cation-binding proteins (PCaPs) (Ide *et al.*, 2007; Nagasaki-Takeuchi *et al.*, 2008). Arabidopsis PCaP1 incorporates [^{3}H]myristic acid during *in vitro* transcription and translation and interacts with phosphatidylinositol phosphates (PtdInsPs). Furthermore, AtPCaP1 could bind calmodulin in a Ca^{2+}-dependent manner (Nagasaki *et al.*, 2008). Hence, AtPCaP1 could be involved in intracellular signaling via interaction with PtdInsPs and calmodulin (Kato *et al.*, 2010).

The 25 kDa protein AtPCaP1 is also called microtubule-destabilizing protein 25 (MDP25) and was found to regulate the elongation of hypocotyl cells by destabilizing cortical microtubules dependent on calcium (Li *et al.*, 2011). When cytoplasmic calcium increases, MDP25 binds directly to and destabilizes microtubules to enhance its depolymerization (Qin *et al.*, 2012). In addition, AtPCaP1 can interact with the movement-required novel protein P3N-PIPO of Turnip mosaic virus (TuMV) and promote viral cell-to-cell movement (Vijayapalani *et al.*, 2012). Disrupted expression of AtPCaP1 reduced the accumulation and cell-to-cell movement of TuMV, which led to enhanced plant resistance.

In this study, we investigated one gene with upregulated expression from a previous study of BaMV inoculation (Cheng *et al.*, 2010) that may play a negative role in regulating the accumulation of BaMV. This gene, designated *NbPCaP1L*, is an ortholog of *PCaP1* in *N. benthamiana*. BaMV accumulation was increased when *NbCaP1L* expression was knocked down but was decreased when *NbCaP1L* was transiently overexpressed. Further analysis indicated that NbCaP1L acts on the movement of BaMV. The mechanism of this regulation is discussed.

Materials and methods

Viruses and plants

BaMV, PVX, and Cucumber mosaic virus (CMV) were used for inoculation. *N. benthamiana* were grown at 28°C in a growth room with a 16 h light/8 h dark cycle.

Virus particle purification

The infected leaves were homogenized with extraction buffer of 0.5 M borate at pH 9.0, 1 mM EDTA, and 0.1% β-mercaptoethanol, in a 1 g/2 ml ratio, filtrated through a miracloth, and centrifuged at 12 000×g at 4°C for 10 min. The supernatant was stirred at 4℃ for 10 min with the addition of 4M K$_2$HPO$_4$ and 2M CaCl$_2$ to 1% and 2%, respectively, and was centrifuged at 12 000×g at 4°C for 10 min. The supernatant was stirred at 4℃ for 30 min with the addition of triton X-100 and PEG 6000 to 2% and 6%, respectively, and was centrifuged at 12 000×g at 4°C for 10 min. The pellet was resuspended in BE buffer of 50 mM borate at pH 8.0 and 1 mM EDTA, and centrifuged at 14 300×g at 4°C for 1 h on a cushion of 20 % sucrose. The virus pellet was resuspended in 10 ml BE buffer and quantification carried out using a spectrophotometer.

Virus-induced gene silencing (VIGS) and virus challenge

Tobacco rattle virus (TRV)-based VIGS was used to knock down the expression of *NbPCaP1L*. The cDNA fragment ACGT11 derived from NbPCaP1L was obtained from cDNA-AFLP and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) previously (Cheng *et al.*, 2010). The 118 bp cDNA was isolated by digestion with *Eco*RI and subcloned into the pTRV2 vector. The resulting plasmid was electroporated into the *Agrobacterium tumefaciens* C58C1 for knockdown experiments as described previously (Huang *et al.*, 2013). Approximately 500 ng of viral particles was mechanically inoculated into the fourth leaf above the infiltrated leaves at 10 d post-infiltration (dpi). Total proteins were extracted from these virus-inoculated leaves at 1, 3, 5, and 7 dpi with BaMV inoculation and 5 dpi with CMV and PVX inoculation.

Cloning the NbPCaP1L gene

To clone the full-length *NbPCaP1L* gene, the 3′ primer (5′ *CTCGAG*TCAGTCG ACAGCTTTTGGTGGTTCCGGT 3′) and 5′ primer (5′ T*GCTAGC GGATCC*AT GATGGGTTATTGGCAAGC 3′) were used for PCR. The 3′ primer contains the *Xho*I site (underlined) and the 5′ primer contains the *Nhe*I and *BamH*I sites (underlined) for cloning. Full-length *NbPCaP1L* was cloned into the pGEM-T Easy vector (Promega). The sequence was verified by sequencing on an IR^2 System (LI-COR Biosciences, Lincoln, NE, USA). Finally, the full-length *NbPCaP1L* gene was subcloned into the pBIN-OFP vector containing orange fluorescent protein (OFP) driven by the CMV 35S promoter (reconstructed from pmKO2-S1; MBL international, Woburn, MA, USA).

Protoplast isolation and inoculation

Protoplasts were isolated from the fourth leaf above *N. benthamiana* leaves infiltrated with TRV/Luc, containing part of the luciferase gene from nucleotide 110 to 508, and TRV/*NbPCaP1L* at 10 d post Agro-infiltration. The detailed protocol for protoplast isolation was previously described (Tsai *et al.*, 1999). In brief, approximately 4 g of tissue was digested with 25 ml of enzyme solution at 25°C overnight. Intact mesophyll protoplasts were collected from the interphase of the 0.55 M mannitol-MES solution and the 0.55 M sucrose cushion. Finally, protoplasts were washed and re-suspended in mannitol-MES solution. Approximately 2.5×10^5 protoplasts were inoculated with 1 μg BaMV RNA with 20% polyethyleneglycol-6000. The inoculated protoplasts were incubated at 25°C under constant light. Total protein and RNA were extracted from protoplasts after 24 h of incubation.

Western blot assay

Total protein harvested from inoculated protoplasts or leaves was separated in 12% SDS-PAGE and transferred to nitrocellulose membranes (PROTRAN BA 85 Schleicher & Schnell), which were probed with the rabbit antibodies for BaMV, PVX, or CMV CP, then incubated with IRDye 800-conjugated affinity-purified anti-rabbit IgG antibody (Rockland Immunochemicals, Gilbertsville, PA, USA). The fluorescence density on membranes was determined using LI-COR Odyssey (LI-COR Biosciences).

RNA extraction and real-time quantitative RT-PCR

Inoculated leaves of *NbPCaP1L* knockdown *N. benthamiana* were collected and ground. Total RNA was extracted as described (Lin *et al.*, 2007). First-strand cDNA was synthesized with 20 pmole 39d(T) oligo primer and reverse transcriptase (Promega) according to the manufacturer's protocol. Real-time quantitative PCR was performed in a 20 μl-reaction containing a 1000X dilution of SYBR green I (Cambrex Bio Science Rockland, ME, USA) with primer GT11_5′ (5′- AAGGTTGTTCCAAAATTAAAGC-3′) and

GT11_3′ (5′- TTCAATCCTGAAACCTTTGGTCCC-3′) in 0.2-ml PCR tubes. The conditions began with an initial hold at 95°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec. The expression of β-actin was amplified with the primer pair actin_5′ (5′ GATGAAGATACTCACAGAAAGA 3′) and actin_3′ (5′ GTGGTTTCATGAATGCCAGCA 3′) as an internal control for normalization.

Detection of BaMV cell-to-cell movement

Approximately 10 d after VIGS infiltrated with TRV/*luciferase* or TRV/*NbPCaP1L*, the fourth and fifth leaves above infiltrated leaves of *N. benthamiana* plants were mechanically inoculated with 7.5 μg pCBG (Lin *et al.*, 2006), the infectious cDNA clone carrying the green fluorescent protein (GFP) gene driven by the BaMV subgenomic promoter. The fluorescence derived from GFP accompanied by BaMV movement was captured by inverted fluorescent microscopy (Olympus IX71) with an excitation wavelength of 460 to 495 nm, an emission wavelength of 510 nm, and a dichromatic mirror at 505 nm at 6 dpi. The area of each lesion was measured using Image J (http://rsbweb.nih.gov/ij/).

Laser scanning confocal microscopy

Plasmids pBIN-OFP (vector only) and pBIN-NbPCaP1L-OFP were transformed into *A. tumefaciens* C58C1. *A. tumefaciens* containing pBIN-OFP, pBIN-NbPCaP1L-OFP or pBIN61-HcPro was cultured to OD600=1; and the cells were resuspended in 10 mM $MgCl_2$ with 500 μM acetosyringone for induction after being spun down. The pBIN-OFP or pBIN-NbPCaP1L culture was mixed with that of pBIN61-HcPro in a 1:1 volume ratio and infiltrated into *N. benthamiana* leaves. The images were taken by laser scanning confocal microscopy (Olympus Fluoview FV1000) with 405 nm and 543 nm laser excitation for cyan fluorescent protein and OFP, respectively, 2 d after infiltration.

Yeast two-hybrid interaction

The gene fragments encoding the MP and CP of BaMV were constructed into the prey plasmid pYESTrp2 and designated pYES-TGBp1, -2, -3, and -CP. The replication-related DNA fragments were constructed into the bait plasmid pHybLex/Zeo and designated pLEX-Capping, -RdRp, and -Helicase (Cheng *et al.*, 2009; Lee *et al.*, 2011). The DNA fragment for cloning *NbPCaP1L* into yeast prey and bait plasmids was amplified with two sets of primers: for the prey plasmid, YES5′PCaP1HindIII (5′ GGG*AAGCTT*ATGATGGGTTATTGGCAAGC 3′) and YES3′PCaP1XhoI (5′ *GTCGAC*TCAGTCGACAGCTTTTG GTGGTTCCGGT 3′), and for the bait plasmid, LEX5′P CaP1EcoRI (5′ *GAATTC*ATGATGGGTTATTGGCAAGC 3′) and LEX3′PCaP1PstI (5′ G*CTGCAG*TCAGTCG ACAGCTTTTGGTGGTTCCGGT 3′) (restriction enzyme site underlined). The amplified DNAs were cloned into pGEM-T Easy vector (Promega) and verified by sequencing. The full-length *NbPCaP1L* was then subcloned into the yeast prey plasmid with *Hin*dIII and *Xho*I sites and into the bait plasmid with *Eco*RI and *Pst*I sites. *Saccharomyces cerevisiae* strain L40, harboring the bait plasmid (pLEX-Capping, -RdRp, or -Helicase) or prey plasmid (pYES-TGBp1, -2, -3, and -CP), was transformed with the prey plasmid pYES-NbPCaP1L or bait plasmid pLEX-NbPCaP1L, respectively. Positive colonies were grown on Trp⁻/His⁻/Zeocin selection agar plates.

Co-immunoprecipitation

N. benthamiana leaves were infiltrated with the Agrobacteria mixture containing the plasmids pKBRepHA21 plus pEpyon-OFP or pKBRepHA21 plus pBIN-NbPCaP1L-OFP. Total proteins were extracted using an extraction buffer of 20 mM Tris–HCl at pH 7.5,

2 mM MgCl$_2$, 300 mM NaCl, 5 mM DTT, and 1% protease inhibitor cocktail (Roche), at 3 dpi. The supernatant was collected after centrifugation at 4000×g at 4°C for 10 min and underwent immunoprecipitation with anti-HA antibody (Sigma-Aldrich, H9658) at 4°C for 4 h. The interacting proteins were incubated with 20 µl protein A magnetic beads (GE Healthcare) at 4°C for 2 h. Finally, the samples were washed, eluted, and underwent western blot analysis with anti-HA (Sigma-Aldrich, H6908), anti-OFP, or anti-BaMV CP antibodies.

Results

Cloning the plasma membrane–associated cation-binding protein 1-like gene NbPCaP1L from N. benthamiana

A cDNA fragment *ACGT11* 118 bp in length was found upregulated after BaMV inoculation by cDNA-AFLP and was revealed to have an effect on BaMV accumulation in *N. benthamiana* (Cheng *et al.*, 2010). This 118 bp cDNA fragment had 94% identity to that of PIP from *N. tabacum* (GenBank accession Y08609.1). This PIP was defined as a new family of PIP because it lacks a transmembrane domain, whereas conventional PIPs have six membrane-spanning domains with cytosolic N- and C-termini (Lin *et al.*, 2011; Zhao *et al.*, 2013). In a further analysis, PIP from *N. tabacum* showed 57% identity to the PCaP1 of *Arabidopsis thaliana* (GenBank accession NM_118145) (see Supplementary Fig. S1 at *JXB* online). The identity of this PIP from *N. tabacum* is more similar to PCaP1 than conventional PIP.

To clone the full-length *ACGT11* from *N. benthamiana*, we designed primers according to the conserved region among the PCaP1 orthologs from different species (Supplementary Fig. S1) and the nucleotide sequence of PIP from *N. tabacum*. The coding sequence of the PCaP1-like gene from *N. benthamiana*, designated *NbPCaP1L* (GenBank accession MF346700), is 711 nucleotides long encoding a 236-amino acid polypeptide and has 89% and 88% nucleotide and amino acid identity to *NtPIP* (Supplementary Fig. S1).

Reducing the expression of NbPCaP1L increases BaMV accumulation in N. benthamiana *plants but not protoplasts*

To characterize the role of *NbPCaP1L* in BaMV infection, we used TRV-based VIGS to knock down the expression of *NbPCaP1L* and inoculate BaMV. Knockdown of *NbPCaP1L* expression by 70% compared with control plants, *Luc*-knockdown plants, as determined by real-time qRT-PCR (Fig. 1A), conferred no morphologic alterations (Supplementary Fig. S2). After normalization with the large subunit of RuBisCO stained with Coomassie blue, western blot analysis revealed that BaMV CP levels increased in *NbPCaP1L* knockdown plants to 180% that of control plants at 5 dpi but not at earlier infection times (Fig. 1B). Furthermore, BaMV levels were strikingly increased up to 2.9-fold in *NbPCaP1L* knockdown plants at 7 dpi (Fig. 1B). Hence, *NbPCaP1L* may suppress the accumulation of BaMV CP in plants, especially during late infection.
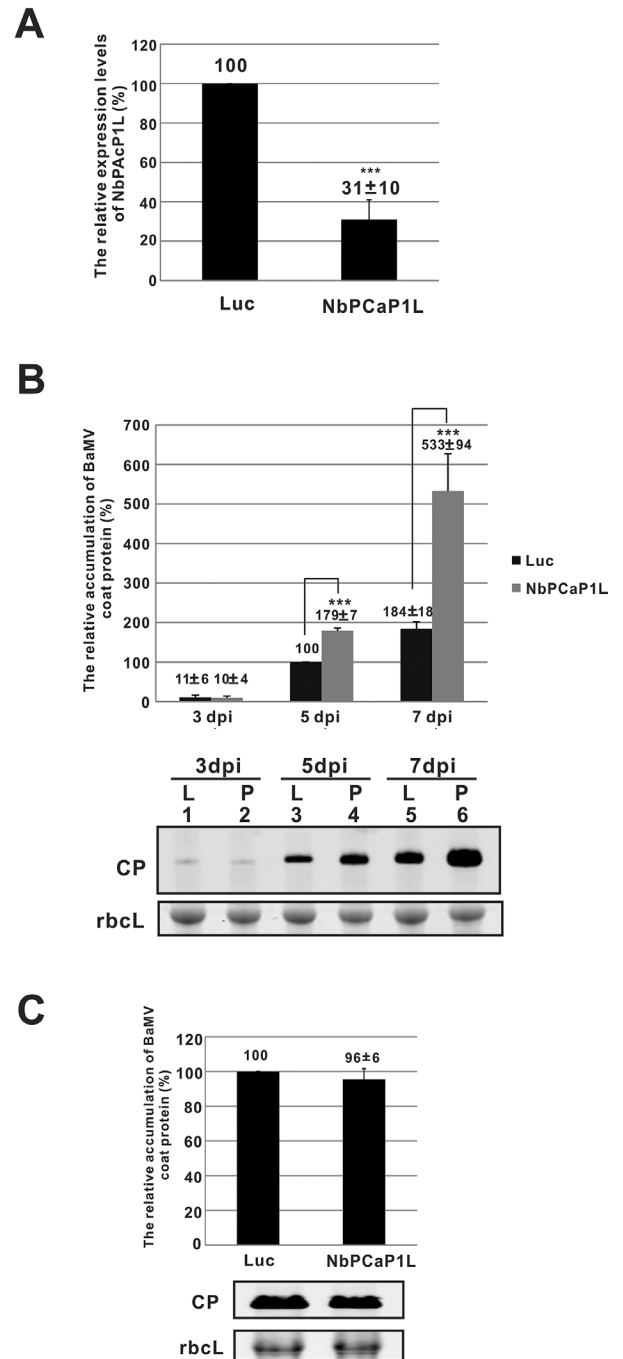
Fig. 1. The relative accumulation of BaMV coat protein in *NbPCaP1L* knockdown plants and protoplasts. (A) The expression of *NbPCaP1L* mRNA was determined by real-time quantitative RT-PCR in *Luc* and *NbPCaP1L* knockdown leaves. (B) Western blot analysis of coat protein (CP) accumulation on the inoculated leaves. The accumulation of control plants was set to 100%. Data are mean ± standard deviation relative levels of BaMV CP from at least three independent experiments with at least three plants in each experiment. Representative western blot results of CP levels with triplicate results and rbcL as a control. (C) Western blot analysis of CP accumulation. The accumulation in control protoplasts at 24 h post inoculation (hpi) was set to 100%. Representative results are shown. Data are mean ± standard deviation relative levels of BaMV CP from at least three independent experiments. Luc, luciferase knockdown control plants or protoplasts; NbPCaP1L, NbPCaP1L knockdown plants or protoplasts; CP, BaMV coat protein; rbcL, RuBisCO large subunit (the loading control for normalization). **$P<0.01$, ***$P<0.001$ by Student's *t*-test.

To investigate whether increased BaMV CP accumulation in *NbPCaP1L* knockdown plants was caused by enhanced viral RNA replication or viral movement in plants, we prepared *NbPCaP1L* knockdown protoplasts to exclude the movement effect. *NbPCaP1L* knockdown and control protoplasts showed no difference in BaMV accumulation (Fig. 1C). Hence, reducing the expression of *NbPCaP1L* did not impede the replication process of BaMV RNA. *NbPCaP1L* could be involved in regulating BaMV cell-to-cell movement. To determine whether reduced *NbPCaP1L* expression could affect other viruses, we inoculated *NbPCaP1L* knockdown plants with PVX and CMV. *NbPCaP1L* knockdown and control plants did not differ in CP accumulation of these viruses at 5 dpi (Supplementary Fig. S3). The effect of *NbPCaP1L* on viral accumulation may be specific to BaMV but not PVX or CMV.
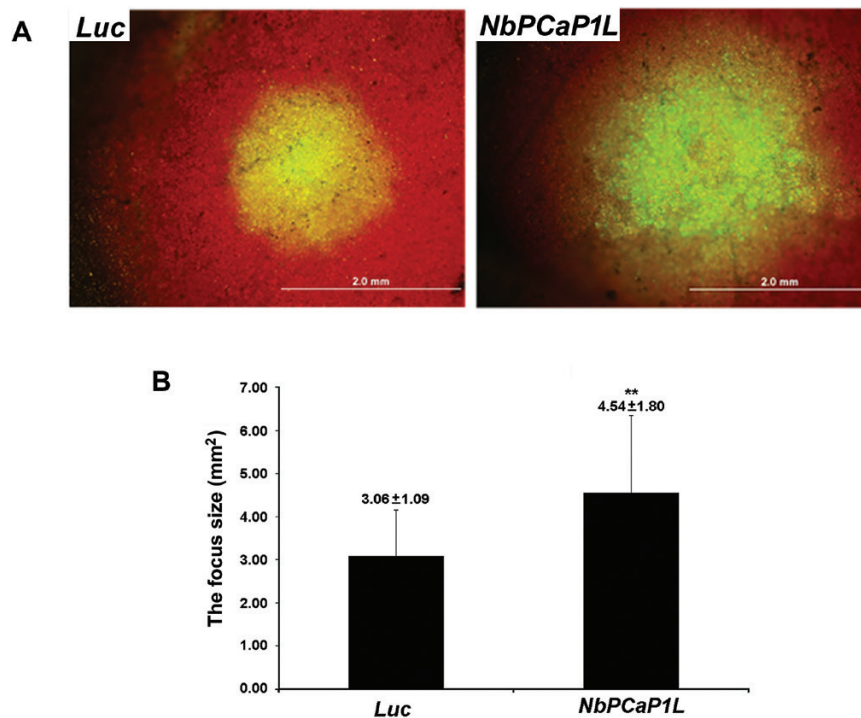
Fig. 2. Cell-to-cell movement of BaMV in *Luc* and *NbPCaP1L* knockdown plants. (A) Fluorescent microscopy of the area of fluorescent foci in inoculated leaves of luciferase knockdown control (*Luc*) and *NbPCaP1L* knockdown (*NbPCaP1L*) plants after inoculation with the BaMV infectious plasmid carrying green fluorescent protein (GFP). Scale bar, 2 mm. (B) Statistical analysis. Data are mean ± standard deviation of 19 and 29 foci from *Luc* and *NbPCaP1L* knockdown plants, respectively. **$P<0.01$ by Student's *t*-test.
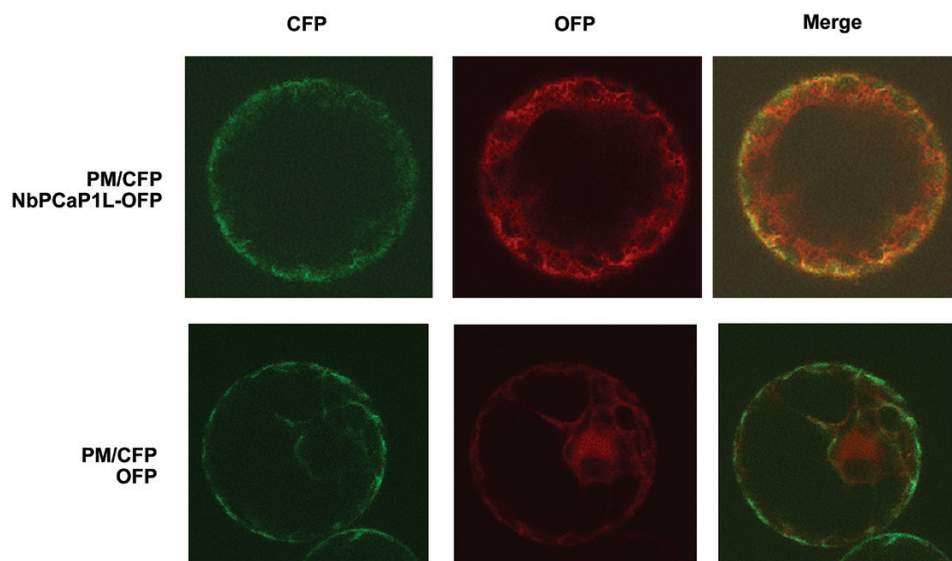
Fig. 3. The localization at the plasma membrane (PM) and cytoplasm of NbPCaP1L with OFP fused at the C-terminus and transiently expressed in *N. benthamiana* protoplasts. Cyan fluorescence protein (CFP) fused with a plasma membrane aquaporin AtPIP2A is transiently expressed in protoplasts and is used as the PM marker.

NbPCaP1L regulates BaMV cell-to-cell movement

As the results of the *NbPCaP1L* knockdown experiments indicated that *NbPCaP1L* had no effect on BaMV accumulation in protoplasts but a marked effect in plants, *NbPCaP1L* may be involved in BaMV cell-to-cell movement. To test this, *NbPCaP1L*- and *Luc*-knockdown plants were inoculated with the full-length infectious BaMV cDNA plasmid pCBG expressing GFP with the BaMV subgenomic promoter (Lin *et al.*, 2004), with measurements taken at 6 dpi (Fig. 2A). The mean GFP focus area in *Luc* and *NbPCaP1L* knockdown plants was 3.06 ± 1.09 and 4.54 ± 1.80 mm^2, respectively (Fig. 2B). Reducing the expression of *NbPCaP1L* in

N. benthamiana plants made them more accessible to BaMV infection, with more and larger foci than in control plants.

BaMV accumulation is reduced in NbPCaP1L-expressing leaves

To visualize the subcellular localization of NbPCaP1L protein in cells, we fused OFP at the C-terminus of NbPCaP1L and transiently expressed it in *N. benthamiana* plants. NbPCaP1L-OFP was clearly present in both the plasma membrane, as it co-localized with a plasma membrane marker, and cytoplasm in protoplasts (Fig. 3). Further analysis of protein extracts after fractionation revealed that NbPCaP1L-OFP was equally
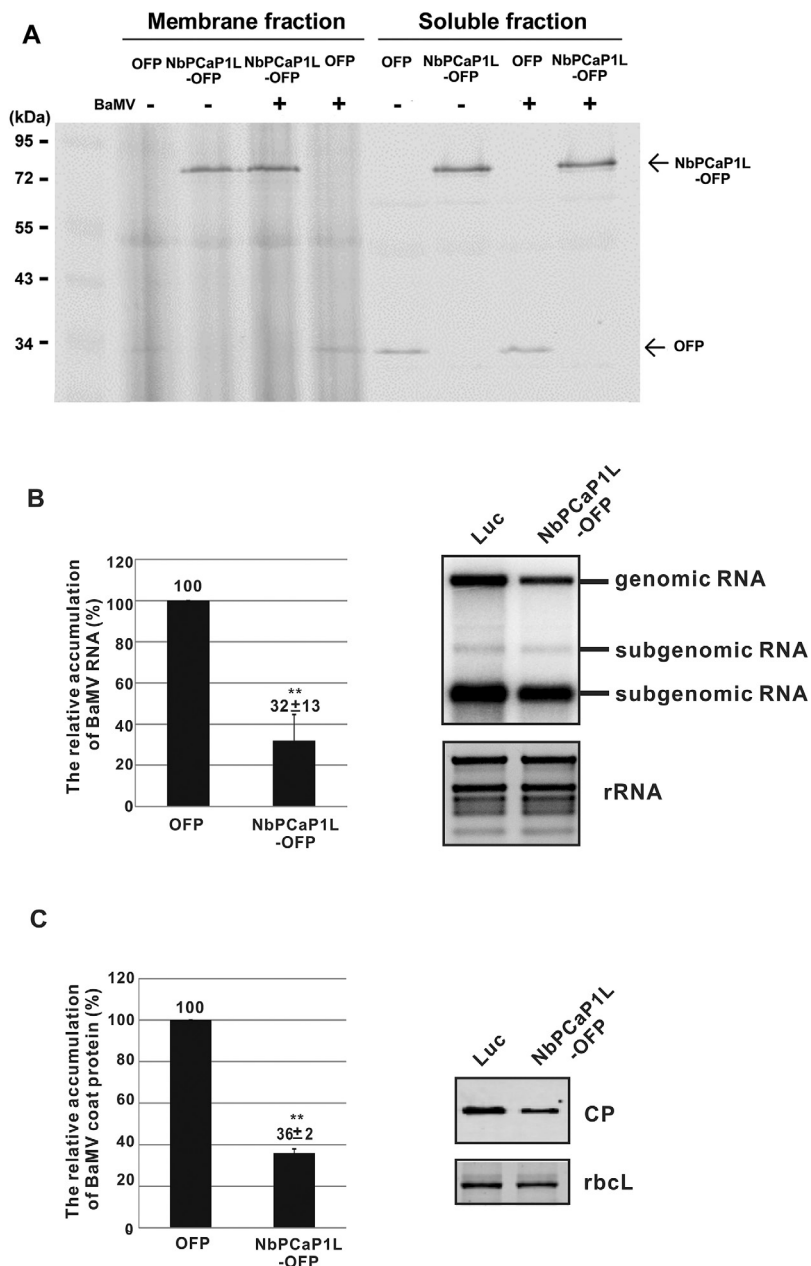
Fig. 4. Effect of expression of *NbPCaP1L* on BaMV infection. (A) Western blot analysis of the fractionation of transiently expressed NbPCaP1L-OFP and OFP alone. Relative accumulation of BaMV CP (B) and RNAs (C) at 5 d post-inoculation (dpi) on inoculated leaves, with NbPCaP1L-OFP or OFP only (as a control) transiently expressed on the same leaves at 3 dpi. Data are mean ± standard error of at least three independent experiments. **$P < 0.01$ by Student's *t*-test.

distributed in the membrane and soluble fractions. This distribution profile of NbPCaP1L-OFP in *N. benthamiana* cells in the membrane and soluble fraction was not changed after BaMV infection (Fig. 4A). The subcellular localization of AtPCaP1 was reported to target the cytoskeleton, cell junction, plasmodesmata, and cell membrane via lipid-anchoring (Nühse *et al.*, 2003; Marmagne *et al.*, 2004; Ide *et al.*, 2007; Nagasaki *et al.*, 2008; Li *et al.*, 2011; Vijayapalani *et al.*, 2012). AtPCaP1 shuttled from the plasma membrane to cytoplasm with increased calcium levels (Kato *et al.*, 2010; Li *et al.*, 2011). The subcellular localization of NbPCaP1L-OFP in both the cytoplasm and plasma membrane was therefore similar to that of AtPCaP1.

To validate whether NbPCaP1L plays a negative role in BaMV infection, we inoculated NbPCaP1L-OFP transiently expressing leaves with BaMV. The BaMV RNA and CP levels were significantly reduced to 32% and 36% of that of control plants, respectively (Fig. 4B, C). Hence, NbPCaP1L negatively regulates BaMV movement.
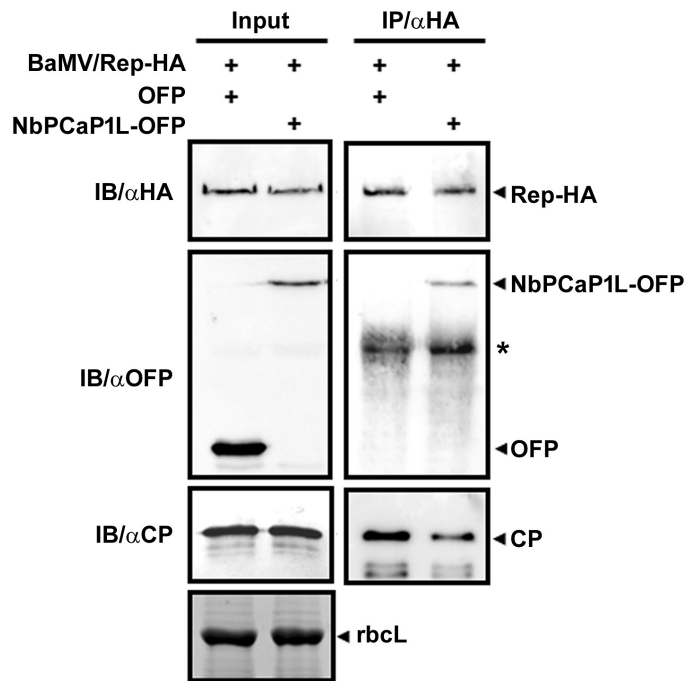
NbPCaP1L interacts with BaMV replicase

To examine whether the host protein NbPCaP1L interacts with any viral-encoded proteins, we used the yeast two-hybrid system. The genes encoding the MPs and CP of BaMV were constructed in a yeast prey plasmid; full-length NbPCaP1L was cloned into the bait plasmid and transformed into yeast containing each of the viral-encoding genes, TGBp1, 2, 3, and CP. No yeast could grow on the selective medium (Trp⁻/His⁻/Zeocin) except the positive control yeast (Fig. 5A).

Another set of constructs containing each domain of the BaMV-encoded replicase, namely the capping, helicase-like, and RdRp domains, was cloned into the bait plasmid of the yeast two-hybrid system (Cheng *et al.*, 2009; Lee *et al.*, 2011). NbPCaP1L was then constructed into the prey plasmid for testing of the interaction with BaMV replicase. The capping and helicase-like domains could interact with NbPCaP1L (Fig. 5B). Overall, these results suggest that the host protein NbPCaP1L could interact with the N-terminal portion of BaMV replicase, that is the capping and helicase-like domains, in the yeast cells.
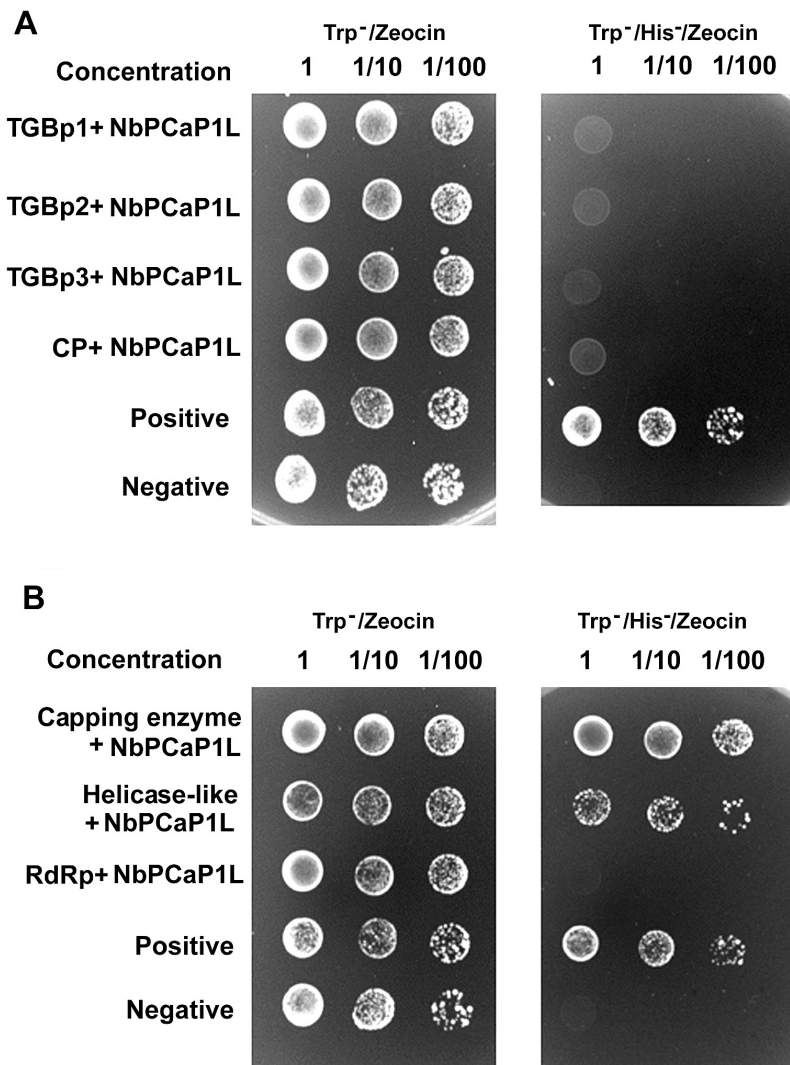
Fig. 5. Interaction of NbPCaP1L with BaMV-encoded polypeptides in yeast cells. Yeast strain L40 co-transformed with the prep plasmid, pYES-TGBp1, -2, -3, or -CP, and bait plasmid pLEX-NbPCaP1L in (A) or prey plasmid pYES-NbPCaP1L and bait plasmid, pLEX-Capping, -RdRp, or -Helicase in (B). Positive control, yeast containing pLEX-Fos2 and pYES-Jun; negative control, yeast containing pHybLex/Zeo and pYESTrp2. Positive colonies were grown on Trp⁻/His⁻/Zeocin selection agar plates. The yeast concentrations with the dilution factor are indicated at the top of each panel.

Fig. 6. Co-immunoprecipitation (IP) assay to verify the interaction between BaMV replication protein and NbPCaP1L. Total proteins (input) were extracted from *N. benthamiana* leaves co-infiltrated with Agrobacteria containing the infectious BaMV/Rep-HA and OFP vector or NbPCaP1L-OFP. Equal amounts of input proteins were used for immunoblotting (IB) with antibodies against HA, OFP, and BaMV CP as indicated. rbcL, RuBisCo large subunit is used as the loading control. After IP with anti-HA antibody, the co-IP of Rep-HA, NbPCaP1L-OFP, and CP was detected using corresponding antibodies for IB.

To validate the results of the yeast two-hybrid finding that NbPCaP1L interacts with BaMV replicase, we used co-immunoprecipitation. BaMV modified with a HA-tag at the C-terminus of the replicase, BaMV/Rep-HA, was agro-infiltrated onto transiently expressed OFP or NbPCaP1L-OFP leaves. Total proteins were immunoprecipitated with anti-HA antibody. NbPCaP1L-OFP could interact with replicase-HA but not OFP alone (Fig. 6).

Discussion

The process of viral movement is critical for plant viruses to establish a successful infection, even with effective replication in cells. Here we investigated whether one host gene from *N. benthamiana*, a plasma membrane-associated cation-binding protein 1-like protein designated *NbPCaP1L*, which shows upregulated expression after BaMV inoculation, was involved in regulating virus movement.

Plants have evolved a few different strategies, such as post-transcriptional gene silencing (Hammond *et al.*, 2001) and hypersensitive response (Baurès *et al.*, 2008), to protect themselves against pathogen infection. However, pathogens can also develop a counter-strike mechanism to break down the host defense systems, such as virus-encoded silencing suppressors. The host could therefore have evolved some other unidentified defense mechanisms to resist the pathogen infection. These

types of offense/defense strategies must be identified to gain knowledge into plant protection, especially for important crops.

These unidentified defense genes might express their genes differentially during pathogen infection. We previously used cDNA-AFLP to isolate the differentially expressed genes from *N. benthamiana* plants after BaMV infection (Cheng *et al.*, 2010). *NbPCaP1L* was one of the potential upregulated defense genes based on our results in this study, which could act on viral movement.

The movement complex of potexviruses, including PVX and BaMV, was proposed to assemble on the perinuclear endoplasmic reticulum-derived membrane-bound body (Tian *et al.*, 2004; Verchot-Lubicz *et al.*, 2010). The complex, comprising the membrane-associated TGBp2 and TGBp3 translated at the endoplasmic reticulum (Verchot-Lubicz *et al.*, 2010) and ribonucleoparticles containing viral RNA, CP, TGBp1, and replicase (Tian *et al.*, 2004; Lee *et al.*, 2011; Wu *et al.*, 2011; Park *et al.*, 2013), was transported to the endoplasmic reticulum network directly or to actin filaments associated with membrane vesicles toward the PD (Tian *et al.*, 2004). The cellular vesicle formation factor Rab-GTPase activation protein, (NbRabGAP1, regulates BaMV cell-to-cell and systemic movement (Huang *et al.*, 2013), so the membrane vesicle could be involved in trafficking the BaMV movement complex.

The association of viral replicase in the movement complex was first proposed by observing the differential time required for the spread of TMV from primary inoculated cells to secondary cells, at 18 to 20 h post inoculation, and secondary cells to tertiary cells, as 2 to 4 h post inoculation (Kawakami *et al.*, 2004). Viral RNA in primary inoculated cells was suggested to need more time to set up the movement complex for trafficking to secondary cells, whereas the movement complex containing the replicase could initiate replication on entry to secondary cells and speed up the assembly of the movement complex to tertiary cells. The interaction between BaMV CP and replicase is needed for efficient viral movement. Mutations in CP that fail to interact with viral replicase diminish cell-to-cell movement in plants (Lee *et al.*, 2011). The anti-TGBp3 immunopurified movement complex from BaMV-infected *N. benthamiana* harbors endogenous RdRp activity (Chou *et al.*, 2013), so BaMV replicase may be included in the movement complex.

The host protein NbPCaP1L could interact with the replicase of BaMV (Figs 5 and 6), so the mechanism of NbPCaP1L retarding the movement of BaMV could involve competing for replicase availability with the competent viral movement complex. Although we did not observe the interaction between NbPCaP1L with viral membrane-associated MPs TGBp2 and TGBp3 in the yeast two-hybrid system (Fig. 5), which might not be a good way to detect this interaction, NbPCaP1L constraining viral movement via interaction with MPs cannot be ruled out. We did however demonstrate that the interaction of NbPCaP1L with BaMV replicase could be the major effector for restricting virus movement.

Arabidopsis PCaP1 could assist the viral movement of TuMV via interaction with the potyviral protein P3N-PIPO of the viral movement complex to target the plasma membrane and localize to the plasmadesmata (Vijayapalani *et al.*,

2012). By contrast, the homolog protein NbPCaP1L in *N. benthamiana* for BaMV may play a different role in retarding viral movement by interacting with the replication protein in the movement complex. NbPCaP1L is a membrane-associated protein that harbors a novel activity by interacting with the viral RdRp and trapping the viral movement complex to restrict viral cell-to-cell movement.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Sequence alignment and the expression profile of NbPCaP1L after BaMV inoculation.

Fig. S2. The morphology of control and *NbPCaP1L* knockdown plants.

Fig. S3. The relative accumulation of viral coat protein (CP) in *NbPCaP1L*-knockdown and control plants.

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