DEVELOPMENTALLY REGULATED PLASMA MEMBRANE PROTEIN of Nicotiana benthamiana Contributes to Potyvirus Movement and Transports to Plasmodesmata via the Early Secretory Pathway and the Actomyosin System

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The intercellular movement of plant viruses requires both viral and host proteins. Previous studies have demonstrated that the frame-shift protein P3N-PIPO (for the protein encoded by the open reading frame [ORF] containing 5’-terminus of P3 and a +2 frame-shift ORF called Pretty Interesting Potyviridae ORF and embedded in the P3) and CYLINDRICAL INCLUSION (CI) proteins were required for potyvirus cell-to-cell movement. Here, we provide genetic evidence showing that a Tobacco vein banding mosaic virus (TVBMV; genus Potyvirus) mutant carrying a truncated PIPO domain of 58 amino acid residues could move between cells and induce systemic infection in Nicotiana benthamiana plants; mutants carrying a PIPO domain of seven, 20, or 43 amino acid residues failed to move between cells and cause systemic infection in this host plant. Interestingly, the movement-defective mutants produced progeny that eliminated the previously introduced stop codons and thus restored their systemic movement ability. We also present evidence showing that a developmentally regulated plasma membrane protein of N. benthamiana (referred to as NbDREPP) interacted with both P3N-PIPO and CI of the movement-competent TVBMV. The knockdown of NbDREPP gene expression in N. benthamiana impeded the cell-to-cell movement of TVBMV. NbDREPP was shown to colocalize with TVBMV P3N-PIPO and CI at plasmodesmata (PD) and traffic to PD via the early secretory pathway and the actomyosin motility system. We also show that myosin XI-2 is specially required for transporting NbDREPP to PD. In conclusion, NbDREPP is a key host protein within the early secretory pathway and the actomyosin motility system that interacts with two movement proteins and influences virus movement.

The movement of viruses in plants can be divided into three stages: intracellular, intercellular, and long-distance movement (Nelson and Citovsky, 2005; Benítez-Alfonso et al., 2010). Plasmodesmata (PD) are plasma membrane-mediated channels in cell walls that control the intercellular trafficking of micromolecules and macromolecules, including plant viruses (Boevink and Oparka, 2005; Lucas et al., 2009). Plant viruses encode movement proteins (MPs) that can regulate the size exclusion limit (SEL) of PD and mediate virus trafficking between cells.

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dedicated MP. To date, multiple potyviral proteins, including COAT PROTEIN, CYLINDRICAL INCLUSION (CI), HELPER COMPONENT PROTEINASE (HC-Pro), and VIRAL GENOME-LINKED PROTEIN, have been shown to function in the cell-to-cell movement of potyviruses (Nicolas et al., 1997; Rojas et al., 1997; Carrington et al., 1998; Wei et al., 2010).

Viruses of Potyviruses (family Potyviridae), the largest genus of plant-infecting viruses, cause great economic losses to world agriculture production (Fauquet et al., 2005). The potyviral genome is a positive sense, single-stranded RNA of approximately 10 kb in length. It contains a large open reading frame (ORF) encoding a polypeptide that is later processed into 10 mature proteins by three virus-encoded proteases (Riechmann et al., 1992; Fauquet et al., 2005). A +2 frame-shift Pretty Interesting Potyviridae (PIPO) ORF that is embedded within the P3 ORF was recently identified and proposed to produce a P3N-PIPO (for the protein encoded by 5’-terminus of P3 and frameshift PIPO) fusion (Chung et al., 2008; Vijayapalani et al., 2012). The P3N-PIPOs of Turnip mosaic virus (TuMV) and Tobacco etch virus were previously shown to localize at PD, interact with CI in planta, and transport CI to PD in a CI:P3N-PIPO ratio-dependent manner (Wei et al., 2010). Soybean mosaic virus with a mutant PIPO domain failed to cause systemic infection in its host plant (Wen and Hajimorad, 2010). Therefore, the potyvirus P3N-PIPO has been suggested as the classical MP (Tilsner and Oparka, 2012; Vijayapalani et al., 2012).

Viruses recruit host factors for their movement in plants (Chen et al., 2000; Raffaele et al., 2009; Amari et al., 2010; Ueki et al., 2010). Compared with the progresses on plants (Chen et al., 2000; Raffaele et al., 2009; Amari et al., 2010; Tilsner and Oparka, 2012; Vijayapalani et al., 2012). TuMV infection in Arabidopsis (Vijayapalani et al., 2012). The P3N-PIPOs of Turnip mosaic virus (TuMV) and Tobacco etch virus were previously shown to localize at PD, interact with CI in planta, and transport CI to PD in a CI:P3N-PIPO ratio-dependent manner (Wei et al., 2010). Soybean mosaic virus with a mutant PIPO domain failed to cause systemic infection in its host plant (Wen and Hajimorad, 2010). Therefore, the potyvirus P3N-PIPO has been suggested as the classical MP (Tilsner and Oparka, 2012; Vijayapalani et al., 2012).

Many viral MPs have been shown to traffic within plant cells via the early secretory pathway and/or along the actin filaments or microtubules. For example, the early secretory pathway and microtubules were required for GFLV MP trafficking to PD (Laporte et al., 2003). TuMV P3N-PIPO and CI were reported to utilize the early secretory pathway rather than the actomyosin motility system for their trafficking to PD (Wei et al., 2010). Several plant myosin motor proteins have been reported to participate in virus intracellular movement (Wei and Wang, 2008; Harries et al., 2010). Myosins VIII-1, VIII-2, and VIII-B were shown to transport a heat shock protein 70 homolog of Beet yellow virus to PD (Avisar et al., 2008a), but only myosin VIII-1 was needed for the nonstructural protein encoded by viral complementary strand of RNA4 (NSv4) of Rice stripe virus traffic to PD (Yuan et al., 2011). A more recent study has indicated that both the secretory pathway and myosins XI-2 and XI-K were required for TuMV cell-to-cell movement (Agbeci et al., 2013). However, it remains largely unknown how the MP-interacting host factor(s) reach their target sites in cells.

Tobacco vein banding mosaic virus (TVBMV) is a distinct potyvirus mainly infecting solanaceous crops (Tian et al., 2007; Yu et al., 2007; Zhang et al., 2011). In this article, we provide evidence showing the length requirements of the PIPO domains for its function in mediating TVBMV movement and the restoration of the movement-defective TVBMV mutants. We also show the interactions between TVBMV P3N-PIPO and CI and NbDREPP, a developmentally regulated plasma membrane protein in Nicotiana benthamiana, and the route by which NbDREPP traffics to PD. Silencing of NbDREPP expression in N. benthamiana significantly impeded the cell-to-cell movement of TVBMV.
Figure 2. Infectivity of the wild-type and mutant TVBMV in *N. benthamiana* plants. A, Schematic representations of the wild-type and mutant TVBMV with truncated PIPO domains. White boxes represent P3N domains, and gray boxes represent the wild-type and mutant PIPO domains of different lengths. Arrowheads indicate the positions of three introns inserted in the parental virus clone. UTR, Untranslated region. B, Phenotypes of the wild-type and mutant TVBMV observed under normal light (top row) or UV illumination (bottom row) at 7 dpai. Arrows point at the infiltrated leaves. The middle row shows the upper young leaves within the dotted rectangles shown in the images in the top row. The bottom row shows the green fluorescence accumulated in the plants infected with the wild-type and mutant viruses. C, Accumulation of negative sense viral RNA in
RESULTS

Expression of TVBMV P3N-PIPO Fusion in Plants

To determine the form of PIPO expressed in the TVBMV-infected plants, we modified pTVBMV-GFP, an infectious clone of the TVBMV HN39 isolate that carries a GFP gene and can express free GFP (Gao et al., 2012), to produce constructs pCamTVBMV-GFP, which can be inoculated via agroinfiltration, and pCamTVBMV-GFP-HIS3N, which expresses a His tag-labeled P3 (Fig. 1A). Both constructs were agroinfiltrated into leaves of *N. benthamiana* and *Nicotiana tabacum*. By 14 dpi, two unique proteins of approximately 40 and 24 kD, similar to the predicted size of TVBMV (His)_6-P3 and (His)_6-P3N-PIPO, were detected by western-blot assays using a His tag-specific antibody in TVBMV-GFP-HIS3N-infected but not TVBMV-GFP-infected *N. benthamiana* and *N. tabacum* plants (Fig. 1B). This result indicated that both P3 and the P3N-PIPO fusion were produced in the TVBMV-infected plants.

The Length of the PIPO Domain Determines the Ability of TVBMV Mutants to Move Between Cells

The P3N-PIPO ORF of wild-type TVBMV encoded a PIPO domain of 60 amino acid residues (Zhang et al., 2011; Gao et al., 2012). To determine the length requirements of the PIPO domain during TVBMV infection, we relocated the stop codon within the P3N-PIPO ORF to produce four TVBMV mutants with truncated PIPO domains while maintaining the original amino acid sequence of P3. The resulting mutants PIPO7aaSTOP, PIPO20aaSTOP, PIPO43aaSTOP, and PIPO58aaSTOP encoded PIPO domains of 7, 20, 43, and 58 amino acid residues, respectively (Fig. 2A). Because the PIPO domains in different potyviruses varied in length (Supplemental Fig. S1), we also produced three more TVBMV mutants with extended PIPO domains (PIPOPLUS8aa, PIPOPLUS22aa, and PIPOPLUS38aa). These three mutants encoded PIPO domains of 68, 82, and 98 amino acid residues, respectively (Supplemental Fig. S2A). The wild-type and mutant TVBMV were individually inoculated to *N. benthamiana* plants via agroinfiltration. By 7 dpi, plants agroinfiltrated with construct pCamTVBMV-GFP, pCamPIPO58aaSTOP, pCamPIPOPLUS8aa, or pCamPIPOPLUS38aa developed mosaic symptoms in their upper young leaves (i.e. systemic leaves). Under UV illumination, these infected plants showed strong GFP fluorescence in their agroinfiltrated and systemically infected leaves (Fig. 2B; Supplemental Fig. S2B). Plants agroinfiltrated with pCamPIPO7aaSTOP, pCamPIPO20aaSTOP, or pCamPIPO43aaSTOP failed to show any virus symptoms in their systemic leaves, and GFP fluorescence was observed only in the agroinfiltrated leaves (Fig. 2B). The reverse transcription (RT)-PCR results showed that TVBMV RNA had accumulated in the systemic leaves of pCamTVBMV-GFP, pCamPIPO58aaSTOP, pCamPIPOPLUS8aa, or pCamPIPOPLUS38aa-agroinfiltrated plants but was not detected in the systemic leaves of plants agroinfiltrated with pCamPIPO7aaSTOP, pCamPIPO20aaSTOP, or pCamPIPO43aaSTOP by 7 dpi (Supplemental Fig. S3). To determine whether the four truncated mutants could replicate in the agroinfiltrated *N. benthamiana* leaves as well as the parental TVBMV-GFP, we analyzed the accumulation levels of their negative-strand RNA by a northern-blot assay using an RNA probe specific for the TVBMV COAT PROTEIN ORF. The results showed that similar amounts of negative-strand RNA had accumulated in the leaves agroinfiltrated with pCamTVBMV-GFP or one of the four mutant constructs by 3 dpi (Fig. 2C; Supplemental Fig. S4), indicating that the replication of these mutants was not affected by the mutations introduced into the PIPO ORF.

To further investigate the role of P3N-PIPO in TVBMV cell-to-cell movement, Agrobacterium tumefaciens cells harboring pCamTVBMV-GFP, pCamPIPO7aaSTOP, pCamPIPO20aaSTOP, pCamPIPO43aaSTOP, pCamPIPO58aaSTOP, pCamPIPOPLUS8aa, pCamPIPOPLUS22aa, or pCamPIPOPLUS38aa were used to infiltrate *N. benthamiana* leaves. Prior to infiltration, the *A. tumefaciens* cells were resuspended and adjusted to an optical density at 600 nm (OD_{600}) = 0.5 with induction buffer, which were further diluted at a ratio of 1:10,000 to ensure that initial transfection occurred in isolated foci of a single cell to allow cell-to-cell movement assessment. By 3 dpi, the leaves agroinfiltrated with pCamPIPO7aaSTOP, pCamPIPO20aaSTOP, or pCamPIPO43aaSTOP showed GFP green fluorescence in single epidermal cells, while the leaves agroinfiltrated with pCamTVBMV-GFP, pCamPIPO58aaSTOP, pCamPIPOPLUS8aa, pCamPIPOPLUS22aa, or pCamPIPOPLUS38aa showed GFP fluorescence in clusters of approximately eight epidermal cells (Fig. 2, D and E; Supplemental Fig. S2C). This result indicated that TVBMV mutants encoding a PIPO domain of 58 or more amino acid residues were competent in cell-to-cell movement while those encoding a PIPO domain of less than 58 amino acid residues were defective in cell-to-cell movement.

Movement-Defective Mutants Restored Their Ability to Cause Systemic Infection via Mutations

The above infectivity assays showed that mutant PIPO7aaSTOP, PIPO20aaSTOP, or PIPO43aaSTOP failed to cause systemic infection in *N. benthamiana* by 7 dpi.
(Fig. 2B). However, by 14 dpai, some *N. benthamiana* plants agroinfiltrated with pCamPIPO7aaSTOP, pCamPIPO20aaSTOP, or pCamPIPO43aaSTOP developed systemic mosaic symptoms. To investigate the reason for this phenomenon, total RNAs were extracted from the systemically infected leaves for RT-PCR followed by sequencing of the progeny viruses. The results showed that the previously introduced stop codons (TGA, TAG, and TGA) in these mutants had mutated to CGA, TGG, or CGA, resulting in the production of the functional P3N-PIPO. Some progeny viruses were found to contain additional mutations in the other regions within the genomic RNA. Most of these mutations were synonymous mutations and a few were nonsynonymous. To determine the role of these nonsynonymous mutations in movement, we introduced them into constructs pCamPIPO7aaSTOP, pCamPIPO20aaSTOP, and pCamPIPO43aaSTOP, respectively. Agroinfiltration of these new mutants to *N. benthamiana* plants did not produce systemic infection in any assayed plant (data not shown).

To determine the frequency of spontaneous mutations at the introduced stop codon sites in the movement-defective mutants, *N. benthamiana* and *N. tabacum* plants were agroinfiltrated with individual movement-defective mutants and grown at 25°C or 30°C. By 14 dpai, systemic symptoms and GFP green fluorescence were observed in 65% to 77% of *N. benthamiana* and 17% to 35% of *N. tabacum* plants grown at 25°C. Only 8% to 9% of *N. benthamiana* and 2% to 10% of *N. tabacum* plants grown at 30°C showed systemic virus symptoms and GFP green fluorescence (Table I). This result indicated that the frequency of spontaneous mutations was host and temperature dependent. Of the three mutants, the highest mutation frequency was found in PIPO43aaSTOP-infiltred host plants grown under both temperature conditions. The lowest mutation frequency was found in PIPO20aaSTOP-infiltred *N. tabacum* plants (Table I).

**TVBMV P3N-PIPO and CI Interact with DREPP of *N. benthamiana***

Candidates that interacted with TVBMV P3N-PIPO were identified from an *N. benthamiana* complementary DNA (cDNA) library using a yeast (*Saccharomyces cerevisiae*)-two-hybrid assay. Sequence analysis showed that five of the candidates shared 87% amino acid sequence identity with NtDREPP, a developmentally regulated plasma membrane protein of *N. tabacum* (Supplemental Fig. S5A). NbDREPP was predicted to have a long intrinsically disordered (ID) region in its C terminus (Supplemental Fig. S5, B and C). The ID region contained four flexible, surface-exposed, and disorder-promoting residues (Ser, Pro, Glu, and Lys; Marin and Ott, 2014) that accounted for about 43% of the total amino acid in the NbDREPP. The full-length NbDREPP ORF was then cloned into the pGBK7 vector, and the wild-type P3N-PIPO ORF or its mutants were individually cloned into the pGADT7 vector. Plasmids pGADT7-T-antigen and pGBK7-laminin were used as a negative control treatment and pGADT7-T-antigen and pGBK7-murine p53 as a positive control treatment during the assay. Like the yeast cells cotransformed with the positive control plasmids, the yeast cells cotransformed with pGBK7-NbDREPP and pGADT7-P3N-PIPO or pGBK7-NbDREPP and pGADT7-P3N-PIPO88aa gave blue colonies on the culture medium containing synthetic dextrose (SD)/−Trp/−Leu/−5-bromo-4-chloro-3-indolyl-α-β-D-galactopyranoside (X-α-Gal) or SD/−Trp/−Leu/−adenine/−His/+X-α-Gal (Fig. 3A). The yeast cells cotransformed with pGBK7-NbDREPP and pGADT7-P3N-PIPO68aa or pGADT7-P3N-PIPO82aa and pGADT7-P3N-PIPO98aa also gave positive results (data not shown). These results demonstrated that both the wild-type and movement-competent TVBMV P3N-PIPO interacted with NbDREPP.

To further confirm this interaction, we fused NbDREPP or TVBMV P3N-PIPO to the C or N terminus of yellow fluorescent protein (YFP), respectively. The plasmids were then agroinfiltrated into *N. benthamiana* leaves. By 72 hpi, YFP yellow fluorescence was observed in NbDREPP-YC (for YFP C-terminus) and P3N-PIPO-YN (for YFP N-terminus)- or P3N-PIPO58aa-YN-coinfiltred *N. benthamiana* cells using a confocal microscope (Fig. 3B). The *N. benthamiana* cells coinfiltred with NbDREPP-YC and P3N-PIPO68aa-YN, P3N-PIPO82aa-YN, or P3N-PIPO98aa-YN also showed YFP yellow fluorescence.

| Mutants       | 25°C  | 30°C  |
|---------------|-------|-------|
|               | No. of Diseased/Inoculated Plants | Frequency of Spontaneous Mutations | No. of Diseased/Inoculated Plants | Frequency of Spontaneous Mutations |
| *N. benthamiana* |       |       |       |       |
| PIPO7aaSTOP   | 29/44 | 65.9  | 3/34  | 8.8   |
| PIPO20aaSTOP  | 29/44 | 65.9  | 3/34  | 8.8   |
| PIPO43aaSTOP  | 40/52 | 76.9  | 3/33  | 9.1   |
| *N. tabacum*  |       |       |       |       |
| PIPO7aaSTOP   | 8/38  | 21.1  | 2/40  | 5.0   |
| PIPO20aaSTOP  | 7/40  | 17.5  | 1/41  | 2.4   |
| PIPO43aaSTOP  | 14/40 | 35.0  | 4/41  | 9.8   |
Figure 3. Interaction between NbDREPP and P3N-PIPO or Cl. A, Growth of yeast cells cotransformed with BD-NbDREPP and AD-P3NPIPO, AD-P3N-PIPO58aa, AD-P3N-PIPO43aa, AD-P3N-PIPO20aa, or AD-P3N-PIPO7aa on a low-stringency medium (SD/-Trp/-Leu/+X-a-Gal) or on a high-stringency selective medium (SD/-Trp/-Leu/-adenine/-His/+X-a-Gal). Yeast cells cotransformed with AD-T-ant and BD-lam or AD-T-ant and BD-p53 were used as negative or positive controls, respectively. AD, Activation domain; BD, binding domain; Lam, human lamin C; p53, murine p53; T-ant, Simian virus 40 large T-antigen. B, BIFC detection of interactions between NbDREPP and P3N-PIPO, P3N-PIPO58aa, P3N-PIPO43aa, P3N-PIPO20aa, or P3N-PIPO7aa in N. benthamiana epidermal cells. Images were captured using a confocal microscope at 72 h post agroinfiltration (hpai). Bars = 20 μm. C, Images of N. benthamiana cells coexpressing NbDREPP-YC and CI-YN, NbDREPP-YN and CI-YC, CI-YN and YC, NbDREPP-YC and YN, or CI-YN and YC at 72 hpai. Bars = 10 μm. D, Coimmunoprecipitation of NbDREPP and CI.
coimmunoprecipitated with TVBMV CI (Fig. 3D).

c-myc. The results showed that NbDREPP was indeed
determined through a coimmunoprecipitation assay.

NbDREPP and CI itself.

results indicated that TVBMV CI could interact with
and YN, or CI-YN and YC (Fig. 3C). Therefore, the BiFC
expressing the negative controls plasmids, NbDREPP-YC
yellow
and CI-YC, or CI-YN and CI-YC (Fig. 3C). No YFP

coexpressing NbDREPP-YC and CI-YN, NbDREPP-YN

yellow
172 hpai, YFP yellow
complementation (BiFC). Our results showed that by
2 hpai, YFP yellow fluorescence was observed in the leaf
cells coexpressing NbDREPP-YC and CI-YN, NbDREPP-YN
and CI-YC, or CI-YN and CI-YC (Fig. 3C). No YFP
yellow

were used as negative controls. The extracted proteins were

filtration followed by total protein extraction.

filtration of these constructs into

filtration, YFP yellow

knocked down through VIGS (Fig. 4B).

The results of fluorescence microscopy showed that
by 2 hpai, TVBMV-GFP infection in the NbDREPP-
silenced N. benthamiana leaves was predominantly
restricted in single cells. However, its infection in the
NbANK-silenced or TRV empty vector-infected N. benthamiana leaves had expanded to large foci with numerous cells (Fig. 4, C and D). These results clearly indicated that NbDREPP played an important role in TVBMV cell-to-cell movement in N. benthamiana.

NbDREPP Colocalizes with P3N-PIPO and CI at PD

To determine the subcellular localization pattern of
NbDREPP in planta, we fused the NbDREPP gene to
GFP, YFP, and DsRed to generate constructs pNbDREPP-GFP, pNbDREPP-YFP, and pNbDREPP-DsRed, respectively. After agroinfiltration of these constructs into N. benthamiana leaves, punctate fluorescent bodies were observed near the cell walls in all infiltrated leaves by 48 hpai (Fig. 5A). Under the higher magnifications, the fluorescent signal from the expressed NbDREPP-GFP, NbDREPP-YFP, or NbDREPP-DsRed fusion appeared as paired punctate bodies at both sides of the cell walls (Fig. 5A). When AtPDLP1-DsRed, a PD marker, was coexpressed with NbDREPP-GFP in the same cells, colocalized red and green fluorescence was observed at PD (Fig. 5B). In addition, NbDREPP was unable to interac-
t with AtPDLP1 in a BiFC assay (Supplemental Fig. S6). These results indicated that NbDREPP localized at PD by itself.

When NbDREPP-YC and P3N-PIPO-YN were coex-
pressed with AtPDLP1-DsRed in N. benthamiana leaf
epidermal cells via agroinfiltration, YFP yellow fluores-
cence from the NbDREPP-YC/P3NPIPO-YN complex overlapped with the red fluorescence from AtPDLP1-
DsRed (Fig. 5C), indicating that NbDREPP and P3N-
PIPO interacted at PD. TVBMV CI by itself localized in
the cytoplasm (Fig. 5D). In the presence of P3N-PIPO or
during TVBMV infection, however, CI was found at
PD and formed paired punctate bodies at the cell

Figure 3. (Continued.)

NbDREPP-c-myc and CI-HA were coexpressed in N. benthamiana leaves via agroinfiltration. The N. benthamiana leaves coexpressing NbDREPP-c-myc and the HA tag or CI-HA and the c-myc tag were used as negative controls. At 72 hpai, leaf lysates were immunoprecipitated with anti-c-myc or anti-HA heads. The immunoprecipitates were then analyzed through western-blot assays using anti-HA or anti-c-myc antibody. IB, Immunoblotting; IP, immunoprecipitation.
Figure 4. Infectivity of TVBMV-GFP in NbDREPP- or NbANK-silenced plants. A, Symptoms of TVBMV-GFP in NbDREPP- or NbANK-silenced *N. benthamiana* plants at 5 and 14 dpa. Images were taken under normal light (first row) or UV illumination (third row). Arrows indicate the leaves infiltrated with *A. tumefaciens* cells carrying pCamTVBMV-GFP. The second row shows young leaves within the dotted rectangles in the first row. The fourth row shows the leaves within the dotted rectangles in the third row. B, Detection of NbDREPP and NbANK gene expression by semiquantitative RT-PCR. Lanes 1 and 2 represent the...
walls (Fig. 5E). This finding suggested that TVBMV P3N-PIPO could interact with CI and transport CI to PD. In addition, in the presence of P3N-PIPO or during the infection of TVBMV, NbDREPP and CI accumulated predominantly as punctate bodies at the cell walls (Fig. 5, F and G).

**NbDREPP Traffics to PD via the Early Secretory Pathway**

To investigate the role of the early secretory pathway in NbDREPP intracellular trafficking, we conducted chemical treatment and dominant-negative inhibition assays. Brefeldin A (BFA) was shown to interfere with the intracellular transport of proteins, membrane materials, and soluble cargo between the endoplasmic reticulum (ER) and the Golgi apparatus within the endomembrane system (Nebenführ et al., 2002; Stefano et al., 2006; Robinson et al., 2007; Cheung and de Vries, 2008). Leaves of *N. benthamiana* were treated with BFA followed by confocal microscopy. The results showed that by 3 h post BFA treatment, the PD targeting of NbDREPP-GFP was disrupted, but treatment with dimethyl sulfoxide (DMSO) showed no such effect (Fig. 6, A and B).

In a later study, NbDREPP-GFP was coexpressed with the dominant-negative mutant ADP-ribosylation factor [Arf1(T31N)] or secretion-associated and ras superfamily-related gene-1b [Sar1b(H74L)] in *N. benthamiana* leaves via agroinfiltration. Arf1(T31N) is a GDP-locked mutant of Arf1 that impairs the Golgi-ER (coat protein complex [COP]) transport pathway (Lee et al., 2002; Takeuchi et al., 2002; Xu and Scheres, 2005; Stefano et al., 2006). Sar1b(H74L) is a GTP-restricted mutant of the small GTPase Sar-1b and exerts a dominant-negative effect on the ER-Golgi (COPII) vesicular transport pathway (Takeuchi et al., 2000; daSilva et al., 2004; Robinson et al., 2007; Hanton et al., 2008). Unlike the localization pattern of NbDREPP-GFP coexpressed with c-myc (an empty vector), green fluorescence in the leaf cells coexpressing NbDREPP-GFP and c-myc-tagged Sar1b(H74L) appeared predominantly in the cytoplasm in most cells examined (Fig. 6, A and B). Similar results also were observed in the cells coexpressing NbDREPP-GFP and c-myc-tagged Arf1 (T31N; Fig. 6, A and B). Western-blot assays using a GFP-specific or a c-myc-specific antibody confirmed the accumulation of NbDREPP-GFP and the dominant-negative Arf1(T31N) or Sar1b(H74L) mutant proteins in the cells (Fig. 6C).

**The Actin Cytoskeleton and Myosin XI-2 Are Essential for Trafficking of NbDREPP to PD**

Chemical and protein inhibition assays were used to evaluate the role of the actomyosin system in the intracellular movement of NbDREPP. Treatment of *N. benthamiana* leaves with latrunculin B (LatB), a chemical inhibitor of actin polymerization (Morton et al., 2000), disrupted the accumulation of NbDREPP-GFP at PD in approximately 80% of the *N. benthamiana* cells examined (Fig. 7, A and C), indicating that the actin cytoskeleton was required for NbDREPP targeting to PD. We then transiently overexpressed myosin tails to disrupt the function of endogenous myosins (dominant-negative inhibition; Avisar et al., 2008a, 2008b; Peremyslov et al., 2008; Amari et al., 2011) and analyzed whether the trafficking of NbDREPP to PD was affected. NbDREPP-GFP was coexpressed with the tails of myosin XI-2, VIII-I, or VIII-B in *N. benthamiana* leaves via agroinfiltration. The results showed that the trafficking of NbDREPP to PD was disrupted in about 70% of the cells coexpressing NbDREPP-GFP and c-myc-tagged myosin XI-2 tail but not in the cells coexpressing NbDREPP-GFP and the tails of myosin VIII-I or VIII-B (Fig. 7, B and C). Immunoblot analysis using a GFP-specific or a c-myc-specific antibody confirmed the accumulation of NbDREPP-GFP and various myosin tails in the coagroinfiltrated leaves (Fig. 7D).

**DISCUSSION**

**NbDREPP Interacted with P3N-PIPO and CI to Facilitate TVMBV Intercellular Movement**

Viral MPs need to interact with host factors to mediate virus movement in plants. Multiple host proteins have been identified to interact with viral MPs and to be involved in virus movement in plants (Chen et al., 2000, 2005; Raffaele et al., 2009; Amari et al., 2010, 2011; Perraki et al., 2012). The potyviral CI known as one of the MPs of potyviruses (Carrington et al., 1998) was shown to target PD with the help of P3N-PIPO in a ratio-dependent manner (Wei et al., 2010). Recently, the plasma membrane-locating AtPCaP1 was shown to interact with TuMV P3N-PIPO during TuMV intercellular movement and to localize at the plasma membrane in Arabidopsis protoplasts (Ide et al., 2007; Vijaypalani et al., 2012). AtPCaP1 is a hydrophilic cation-binding protein without a predicted transmembrane domain and is associated with the plasma membrane via
Figure 5. Subcellular localization of NbDREPP, P3N-PIPO, and CI in N. benthamiana epidermal cells. A, Transient expression of NbDREPP-GFP, NbDREPP-YFP, and NbDREPP-DsRed in N. benthamiana leaf epidermal cells. Arrows point to punctate bodies formed with NbDREPP-GFP, NbDREPP-YFP, and NbDREPP-DsRed, a PD marker, in N. benthamiana epidermal cells. Arrows point to PD with both NbDREPP-GFP and AtPDLP1-DsRed. B, Colocalization of NbDREPP-GFP and AtPDLP1-DsRed. C, Coexpression of P3N-PIPO-YN, NbDREPP-YC, and AtPDLP1-DsRed in an N. benthamiana cell at 48 hpi. Arrows point to punctate bodies at PD. DIC, Differential interference contrast. D, Localization of CI-GFP or CI-YFP in the cytoplasm. E, Localization of CI-GFP in the presence of P3N-PIPO (left) or during TVBMV infection (right). Arrows point to CI-GFP punctate bodies at PD.
N-myristoylation at the Gly residue at position 2 (Nagasaki et al., 2008). In this study, we identified that TVBMV P3N-PIPO could interact with NbDREPP, whose homolog was identified previously in *N. tabacum* and was predicted to be a developmentally regulated plasma membrane protein (Logan et al., 1997). NbDREPP shared 52% amino acid identity and a highly conserved N terminus with AtPCaP1 (Supplemental Fig. S5A), suggesting that NbDREPP is a homolog of AtPCaP1. We further showed that NbDREPP localized at PD (Fig. 5, A and B), interacted with both TVBMV P3N-PIPO and CI, and colocalized with P3N-PIPO and CI at PD (Figs. 3 and 5, F and G). It still remains unknown whether AtPCaP1 can localize at PD in epidermal cells and interact with CI.

The subcellular localization of a protein may be affected when it is coexpressed with interacting protein(s). TVBMV CI alone aggregated in the cytoplasm (Figs. 3C and 5D), while NbDREPP localized at PD (Fig. 5A). When coexpressed in *N. benthamiana* leaf epidermal cells, NbDREPP and CI colocalized at the cell periphery

**Figure 5. (Continued.)**
Arrowheads points to CI inclusions in the cytoplasm. CI-GFP formed paired punctate bodies at PD in the presence of P3N-PIPO or during TVBMV infection. F and G, NbDREPP-DsRed and CI-GFP colocalized near the cell wall and in the cytoplasm in the presence of the untagged P3N-PIPO or during TVBMV infection. Arrows point to the NbDREPP-DsRed and CI-GFP punctate bodies. Arrowheads point to lump-like structures in the cytoplasm. All images were taken at 48 hpi. Bars = 10 μm.
Furthermore, in the presence of P3N-PIPO, expressed from a transient expression vector (Fig. 5F) or from the virus (Fig. 5G), CI and NbDREPP were found to colocalize at PD. These results suggested that, in the infection process of TVBMV, P3N-PIPO, CI, and NbDREPP might form a complex at PD to mediate potyvirus movement between cells.

NbDREPP was predicted to be an ID protein with a long ID region in the C terminus (Supplemental Fig. S5, B and C). Most known ID proteins undergo folding upon interaction with their partners (Marín and Ott, 2014). Interaction of NbDREPP with P3N-PIPO and/or CI might induce NbDREPP to undergo disorder-to-order transitions prior to the modification of PD. Similar to the role of AtPCaP1 in TuMV movement, silencing of NbDREPP or StPCaP1 (the homologous gene of AtPCaP1 in tomato [Solanum lycopersicum]) expression through VIGS impeded TVBMV infection (Fig. 4; Supplemental Fig. S8). However, knockdown of AtPCaP1 or NbDREPP expression in plants did not completely inhibit the intercellular movement of TuMV or TVBMV, suggesting that additional host factor(s) must participate in their intercellular movement.

**NbDREPP Targeted to PD via the Early Secretory Pathway and Actomyosin Network**

Although several host intracellular trafficking systems have been reported for virus intracellular trafficking (Lazarowitz and Beachy, 1999; Jackson, 2000; Fedorkin et al., 2001), the molecular mechanism by which viral MPs and their interacting host factors are delivered to the PD remained largely unknown (Harries et al., 2010). For example, targeting PD by *Cowpea mosaic virus* MP or *Poa semilatent virus* TGBp3 was not affected by disrupting the ER-Golgi transport pathway or the cytoskeleton network (Pouwels et al., 2002; Schepetilnikov et al., 2008). In contrast, the MPs of *Beet yellows virus* and *Rice stripe virus* required the participation of class VIII myosins (Avisar et al., 2008a; Yuan et al., 2011). Silencing the myosin XI-2 gene in *N. benthamiana* inhibited the movement of TMV.
but not *Potato virus X, Tomato bushy stunt virus*, or *Turnip vein clearing virus* (Harries et al., 2009). The routes by which viral MPs and their interacting host proteins move to PD may differ. For example, GFLV MP targeted PD through diffusion or trafficking along the microtubules (Laporte et al., 2003), while its interacting protein AtPDLP1 targeted PD via the early secretory pathway in a myosin XI-2- and XI-K-dependent manner (Thomas et al., 2008; Amari et al., 2011). Delivery of TuMV P3N-PIPO and CI to PD also required the early secretory pathway but not the actomyosin motility system (Wei et al., 2010). However, the cell-to-cell movement of TuMV required both a functional secretory pathway and actomyosin network, although the target proteins within these host intracellular transport machineries have not been determined (Agbeci et al., 2013). Here, we showed that the trafficking of NbDREPP to PD depended on the early secretory pathway in a myosin XI-2-dependent manner (Figs. 6 and 7). Like TuMV P3N-PIPO, TVBMV P3N-PIPO also was transported to PD via the early secretory pathway but not the actomyosin motility system (Supplemental Fig. S9). The results suggested that NbDREPP trafficked to PD by a route different from that of P3N-PIPO and formed movement complexes with P3N-PIPO and CI at PD to mediate the intercellular movement of TVBMV. Therefore, NbDREPP is a key host protein within the early secretory pathway and the actomyosin motility system that interacts with two MPs and influences virus movement.

**PIPO Is Expressed as a P3N-PIPO Fusion in TVBMV-Infected Cells and Has Length Variability**

A previous study suggested that the PIPO of TuMV was translated as a P3-PIPO fusion through a ribosomal frame-shifting or a transcriptional slippage strategy (Chung et al., 2008). Later, this fusion was confirmed in TuMV-infected plant tissues through western-blot assays using a P3- and PIPO-specific antibody (Vijayapalani et al., 2012). In this study, we continued this work by fusing a His tag to the N terminus of TVBMV P3. Our result showed that the TVBMV PIPO was indeed expressed as the P3N-PIPO fusion during TVBMV infection (Fig. 1B). Our findings, together with the previous reports, indicated that the P3N-PIPO fusion could be found in plant tissues infected with different potyviruses.

The length of the P3N-PIPO fusion was reported to vary among different potyviruses and even among different isolates of the same species (Chung et al., 2008; Cuevas et al., 2012). Our sequence alignments using the published sequences of *Potato virus Y* and *Plum pox virus* agreed with the above reports (Supplemental Fig. S2). More recently, Hillung et al. (2013) reported that the PIPO length variation was controlled by a host-driven selection mechanism. Our results presented here showed that the TVBMV mutants with a deletion of two amino acid residues or an extension of up to 38 amino acid residues in the PIPO domain were functional in mediating TVBMV movement (Fig. 2; Supplemental Fig. S2). The longest movement-competent TVBMV PIPO domain tested in this study contained 40 more amino acid residues, which account for two-thirds of the wild-type PIPO domain, than the minimum movement-competent PIPO domain. This finding shed new light on the population diversity of potyviral P3N-PIPO.

RNA-dependent RNA polymerase (RdRp) of RNA viruses generates numerous mutations in viral genomes during error-prone RNA replication (Domingo and Holland, 1997; Moya et al., 2004; Lauring and Andino, 2010). Some mutations generated by viral RdRp are...
lethal to viruses, while other mutations may increase the fitness of viruses and lead to viral quasispecies in nature (Domingo and Holland, 1997). Because the truncated mutations in the TVBMV PIPO domain had no influence on viral replication, mutant viruses might continue to produce mutations in this region. Although spontaneous mutations occurring precisely at sites of the introduced stop codons were rare, once a mutation led to an elimination of the introduced stop codon, the progeny viruses would restore systemic movement activity and cause systemic infection. It is noteworthy that temperature and host plants had profound effects on the frequency of spontaneous mutations (Table I). This finding agreed with a previous report showing that host plants and temperature could affect the fidelity of viral RdRp (Pita et al., 2007).

A New Model for NbDREPP and Potyvirus Movement

Combining our data with the results published previously (Wei et al., 2010; Amari et al., 2011; Vijayapalini et al., 2012), we propose a new model for NbDREPP intracellular trafficking and potyvirus cell-to-cell movement (Fig. 8). Unlike the two recently proposed models for potyvirus movement (Wei et al., 2010; Vijayapalini et al., 2012), our model emphasizes that (1) the length of P3N-PIPO is variable and only those that meet the length requirements can mediate potyvirus movement; (2) NbDREPP interacts with both P3N-PIPO and CI at PD, and they are all essential components of the potyviral movement complex; and (3) NbDREPP traffics to PD via the COPI- and COPII-dependent early secretory pathway and is empowered by the motor of myosin XI-2. The results presented here further improved our understanding of the mechanism of potyvirus movement and the discovery of new host resistance against the largest group of plant-infecting viruses.

MATERIALS AND METHODS

Plasmid Construction

The full-length intron-containing TVBMV-GFP sequence was released from plasmid pTVBMV-GFP (Gao et al., 2012) using restriction enzymes Sall and SmaI (New England Biolabs) and inserted into pCambia0350 to generate pCamTVBMV-GFP for agroinfiltration into Nicotiana benthamiana plants. To construct pCamTVBMV-GFP-HIS3N, a TVBMV infectious clone with a His tag-labeled P3, 24 nucleotides for agroinfiltration into Nicotiana benthamiana leaves using needleless syringes. The crude leaf extracts were centrifuged at 20,000g for 15 min, and the supernatants were incubated overnight at 4°C with 5 μg of rabbit anti-HA or anti-c-myc antibody as instructed (Sigma-Aldrich). Fifty-microliter protein A/G plus agarose beads (Santa Cruz Biotechnology) were equilibrated with the extraction buffer and then added to each assay sample. After 4 h of incubation at 4°C, the protein/bead complex was pelleted at 600g for 4 min followed by four washes in a washing buffer (25 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml actoscytoringone, and 10 mM MES) for 3 h at room temperature. Individual A. tumefaciens culture was adjusted to OD600 = 0.5 for protein expression, OD600 = 0.5 for virus inoculation, or as indicated otherwise. The diluted A. tumefaciens cultures were infiltrated individually into leaves of N. benthamiana or N. tabacum using needleless syringes.

Coimmunoprecipitation and Immunoblot Assays

For coimmunoprecipitation assays, agroinfiltrated N. benthamiana leaves were collected and ground in liquid nitrogen. Total proteins from the harvested N. benthamiana leaves were extracted using an extraction buffer containing 25 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10 mM dithiobreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml actoscytoringone, 2% (w/v) polyvinylpolypyrrolidonone, 0.15% (v/v) Nonidet P-40, and 1% protease inhibitor cocktail (Roche). The crude leaf extracts were centrifuged at 20,000g for 15 min, and the supernatants were incubated overnight at 4°C with 5 μg of rabbit anti-HA or anti-c-myc antibody as instructed (Sigma-Aldrich). Fifty-microliter protein A/G plus agarose beads (Santa Cruz Biotechnology) were equilibrated with the extraction buffer and then added to each assay sample. After 4 h of incubation at 4°C, the protein/bead complex was pelleted at 600g for 4 min followed by four washes in a washing buffer (25 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 10% [v/v] glycerol). Proteins in the resulting samples were separated on 10% (w/v) SDS-PAGE gels. After transferring protein bands to nitrocellulose membranes, the membranes were probed with a mouse anti-c-Myc or anti-HA antibody (Santa Cruz Biotechnology). The detection signal was visualized using the Pierce ECL western-blot substrates (Thermo Fisher Scientific).

RT-PCR and Northern Blotting

Total RNAs were extracted from the harvested N. benthamiana leaves using Trizol reagent (TransGen Biotech) and then treated with RNase-free DNase I (Takara). RT was conducted with an oligo(dT)18 primer and the EasyScript first-Strand cDNA Synthesis SuperMix kit (TransGen Biotech). PCR was performed using the specific primers listed in Supplemental Table SI and a Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). The PCR products were visualized on agarose gels through electrophoresis.
For northern-blot assays, the agroinfiltrated *N. benthamiana* leaves were harvested at 3 dpi. Total RNAs were extracted from the harvested leaf tissues, separated on 1% (w/v) denaturing agarose gels containing 2% (v/v) formaldehyde, and transferred to Hybond N+ nylon membranes (GE Healthcare) by capillary action. The membranes were probed for viral RNA using a digoxigenin-labeled riboprobe specific for the TVBMV COAT PROTEIN ORF. The RNA band intensity was analyzed by Quantity One software (Bio-Rad).

**Yeast Two-Hybrid and BiFC Assays**

To identify host protein(s) that interact with TVBMV P3N-PIPO, an *N. benthamiana* cDNA library was constructed and screened using the GAL4 2H1 (Clontech) as instructed. Full-length P3N-PIPO ORF was PCR amplified and cloned into the bait vector pGBK7. Briefly, the library *Saccharomyces cerevisiae* Y187 strain and the AH109 strain harboring pGBK7-P3N-PIPO were mated, plated on the double dropout medium containing X-α-Gal (SD/-Leu/-Trp/+X-α-Gal), and then incubated at 30°C for 3 d. The positive cotransformants were then subjected to high-stringency screenings on the quadruple dropout medium with SD/-Leu/-Trp/-adенинe/-His. The protein-protein interactions were then verified in yeast strain Y2HGold.

For BiFC assays, two fusion constructs were agroinfiltrated into *N. benthamiana* leaves using needleless syringes. Cells expressing the fusion proteins were imaged using a Zeiss LSM510 META laser scanning confocal microscope.

**Pharmacological Assays**

The pharmacological assays were performed as described previously (Brandizzi et al., 2002). To disrupt the actin microfilament, leaves agroinfiltrated with various constructs were infiltrated with 5 μM LatB (Sigma-Aldrich) in 0.1% (v/v) DMSO at 4 hpi before examining with the confocal microscope. To investigate the transport of NbDREPP between the ER and Golgi, the agroinfiltrated *N. benthamiana* leaves were infiltrated with BFA (50 μg mL−1 in 0.1% [v/v] DMSO) at 48 hpi. The BFA-infiltrated treated leaves were allowed to grow for 3 h before confocal microscopic examination. Leaves infiltrated with 0.1% (v/v) DMSO were used as controls for these assays.

**Fluorescence and Confocal Microscopy Observation**

*A. tumefaciens* cultures (OD605 = 0.5) harboring the parental or mutant TVBMV-GFP constructs were diluted with induction buffer at a ratio of 1:10,000 (v/v) DMSO at 40 hpa. The LatB-treated leaves were allowed to grow for 12 h before agroinfiltration. Leaf areas were harvested at 3 dpai. Total RNAs were extracted from the harvested leaf tissues, separated on 1% (w/v) denaturing agarose gels containing 2% (v/v) formaldehyde, and transferred to Hybond N+ nylon membranes (GE Healthcare) by capillary action. The membranes were probed for viral RNA using a digoxigenin-labeled riboprobe specific for the TVBMV COAT PROTEIN ORF. The RNA band intensity was analyzed by Quantity One software (Bio-Rad).

**Sequence Analysis**

The amino acid sequences of NbDREPP and its orthologs were analyzed using Jalview (Waterhouse et al., 2009). Multiple sequence alignments were conducted using ClustalX2 (Larkin et al., 2007). Possible protein disorder was predicted with DISOPRED3 (Monastyrskyy et al., 2013) and MetaDisorderMD2 (Kozlowski and Bujnicki, 2012). The GenBank accession numbers for NbDREPP and its orthologs are as follows: KJ830947 (N. benthamiana), AJZ77965 (N. tabacum), XM000248365 (tomato), and NM01036600 (Arabidopsis).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Amino acid alignment of the PIPO domains of different PVY and PPV isolates.

**Supplemental Figure S2.** Infectivity of the wild-type and mutant TVBMV with extended PIPO domains in *N. benthamiana* plants.

**Supplemental Figure S3.** Detection of the wild-type and mutant TVBMV RNA in the systemic leaves of *N. benthamiana* through RT-PCR at 7 dpi.

**Supplemental Figure S4.** Quantitative analysis of the Northern-blot results using Quantity One software (Bio-Rad).

**Supplemental Figure S5.** Amino acid alignment of NbDREPP with its orthologs and prediction of protein disorder.

**Supplemental Figure S6.** BiFC detection of the interaction between NbDREPP and AtPDPL1.

**Supplemental Figure S7.** Coexpression of CI-GFP and NbDREPP-DsRed in *N. benthamiana* leaf epidermal cells.

**Supplemental Figure S8.** Infectivity of TVBMV-GFP in the *SicCalP1*-silenced *Solium Lycopersicum* plants at 8 dpi.

**Supplemental Figure S9.** Effect of the early secretory pathway and actomyosin system on the PD localization of TVBMV P3N-PIPO.

**Supplemental Table S1.** Sequences of the primers used in this study.

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**LITERATURE CITED**

Agbeci M, Grangeon R, Nelson RS, Zheng H, Laliberté JF (2013) Contribution of host intracellular transport machineries to intercellular movement of turnip mosaic virus. PLoS Pathog 9: e1003683

Amari K, Boulant E, Hofmann C, Schmitt-Keichinger C, Fernandez-Calvino L, Didier P, Lerich A, Mutterer J, Thomas CL, Heinlein M, et al (2010) A family of plasmodesmal proteins with receptor-like properties for plant viral movement proteins. PLoS Pathog 6: e1001119

Avisar D, Prokhnovksy AJ, Dolja VV (2008a) Class VIII myosins are required for plasmodesmal localization of a closterovirus Hep70 homolog. J Virol 82: 2836–2843

Avisar D, Prokhnovksy AJ, Makarova KS, Koonin EV, Dolja VV (2008b) Myosin XI-K is required for rapid trafficking of Golgi stacks, peroxisomes, and mitochondria in leaf cells of *Nicotiana benthamiana*. Plant Physiol 146: 1098–1108
Benitez-Alfonso Y, Faulkner C, Ritzenthaler C, Maule AJ (2010) Plasmodesmata: gateways to local and systemic virus infection. Mol Plant Microbe Interact 23: 1403–1412

Boevink P, Opara KJ (2005) Virus-host interactions during movement processes. Plant Physiol 138: 1815–1821

Brandizzi F, Snapp EL, Roberts AG, Lippincott-Schwartz J, Hawes C (2002) Membrane protein transport between the endoplasmic reticulum and the Golgi in tobacco leaves is energy dependent but cytoskeleton independent: evidence from selective photobleaching. Plant Cell 14: 1293–1309

Carrington JC, Jensen PE, Schaad MC (1998) Genetic evidence for an essential role for potyvirus CI protein in cell-to-cell movement. Plant J 14: 393–400

Chen MH, Sheng J, Hind G, Handa AK, Citovsky V (2005) Interaction between the tobacco mosaic virus movement protein and host cell pectin methylsterases is required for viral cell-to-cell movement. EMBO J 19: 913–920

Chen MH, Tian GW, Gafni Y, Citovsky V (2005) Effects of calreticulin on viral cell-to-cell movement. Plant Physiol 138: 1866–1876

Cheung AY, de Vries SC (2008) Membrane trafficking: intracellular highways and country roads. Plant Physiol 147: 1451–1453

Chung BY, Schaller M, Arnosti JF, Firth AE (2010) Plant Sar1 isoforms with near-identical protein sequences exhibit different localizations and effects on secretion. Plant Mol Biol 67: 283–294

Fedorkin O, Soloyev A, Yelina N, Zamyatnin A Jr, Zinovkin R, Mäkinen LJ, Rytölä K (2010) Plant viruses: invaders of cells and pirates of RNA. New York: Kluwer Academic/Plenum Publishers

Fauquet CM, Mayo M, Maniloff J, Desselberger U, Ball LA (2005) Virus Taxonomy: Classification and Nomenclature of Viruses. VIIIth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego

Gao R, Tian YP, Wang J, Yin X, Li XD, Valkonen JP (2008) Tobacco vein banding mosaic virus (genus Potyvirus). Virus Res 136: 276–281

Hantson SL, Chatte L, Matheson LA, Rossi M, Held MA, Brandizzi F (2008) Plant Sar1 isoforms with near-identical protein sequences exhibit different localizations and effects on secretion. Plant Mol Biol 67: 283–294

Harries PA, Park JW, Sasaki N, Ballard KD, Maule AJ, Nelson RS (2009) Differing requirements for actin and myosin by plant viruses for sustained intracellular movement. Proc Natl Acad Sci USA 106: 17594–17599

Harries PA, Schoelz JE, Nelson RS (2010) Intracellular transport of viruses and their components: utilizing the cytoskeleton and membrane highways. Mol Plant Microbe Interact 23: 1381–1393

Hillung J, Elena SF, Cuevas JM (2013) Intra-specific variability and biological relevance of PSS-PtPc protein length in potyviruses. BMC Evol Biol 13: 249

Ide Y, Nagasaki N, Tomioka R, Suito M, Kамиy台南, Maeshima M (2007) Molecular properties of a novel, hydrophilic cation-binding protein associated with the plasma membrane. J Exp Bot 58: 1173–1183

Jackson AO, Lim HS, Bragg J, Ganesan U, Lee MY (2009) Hordeivirus replication, movement, and pathogenesis. Annu Rev Phytopathol 47: 385–422

Jackson D (2000) Opening up the communication channels: recent insights into plasmodesmal function. Curr Opin Plant Biol 3: 394–399

Kawakami S, Watanabe Y, Beachy RN (2004) Tobacco mosaic virus infection spreads cell to cell as intact replication complexes. Proc Natl Acad Sci USA 101: 6291–6296

Kozloowski LP, Bujnicki JM (2012) MetaDisorder: a meta-server for the prediction of intrinsic disorder in proteins. BMC Bioinformatics 13: 111

Laporte C, Vetter G, Loudes AM, Robinson DG, Hillmer S, Stussi-Garaud C, Ritzenthaler C (2003) Involvement of the secretory pathway and the cytoskeleton in intracellular targeting and tubule assembly of

Grapevine fanleaf virus movement protein in tobacco BY-2 cells. Plant Cell 15: 2058–2075

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948

Lauri M, Anido R (2010) Quasispecies theory and the behavior of RNA viruses. PLoS Pathog 6: e1000105

Lazavorit S, Beachy RN (1999) Viral movement proteins as probes for intracellular and intercellular trafficking in plants. Plant Cell 11: 535–548

Lee MH, Min MK, Lee YJ, Jin JB, Shin DH, Kim DH, Lee KH, Hwang I (2002) ADP-ribosylation factor 1 of Arabidopsis plays a critical role in viral trafficking and maintenance of endoplasmic reticulum morphology in Arabidopsis. Plant Physiol 129: 1507–1520

Lim HS, Bragg JN, Ganesan U, Ruzin S, Schichnes D, Lee MY, Vaira AM, Ryu KH, Hammond J, Jackson AO (2009) Subcellular localization of the barley stripe mosaic virus triple gene block proteins. J Virol 83: 9432–9448

Liu H, Naismith JH (2008) An efficient one-step site-directed deletion, insertion, single and multiple-plasmid mutagenesis protocol. BMC Biotechnol 8: 91

Liu Y, Schiff M, Marathe R, Dinesh-Kumar SP (2002) Tobacco RAR1, EDS1 and NDR1/NIM1like genes are required for N-mediated resistance to tobacco mosaic virus. Plant J 30: 415–429

Logan DC, Domeruge O, Teyssendier de la Serve B, Rossignon M (1997) A new family of plasma membrane polypeptides differentially regulated during plant development. Biochem Mol Biol Int 43: 1051–1062

Lucas WJ (2006) Plant viral movement proteins: agents for cell-to-cell trafficking of viral genomes. Virology 344: 169–184

Lucas WJ, Ham BK, Kim JY (2009) Plasmodesmata: bridging the gap between neighboring plant cells. Trends Cell Biol 19: 495–503

Marin M, Ott T (2014) Intrinsic disorder in plant proteins and phytopathogenic bacterial effectors. Chem Rev 114: 6912–6932

Monastyrskyy B, Krystofachovych A, Moul J, Tramontano A, Fidelis K (2013) Assessment of protein disorder region predictions in CASP10. Proteins 82: 127–137

Morton WM, Ayscough KR, McLaughlin PJ (2000) Latrunculin alters the actin-monomer subunit interface to prevent polymerization. Nat Cell Biol 2: 376–378

Moya A, Holmes EC, González-Candelas F (2004) The population genetics and evolutionary epidemiology of RNA viruses. Nat Rev Microbiol 2: 279–288

Nagasaki N, Tomioka R, Maeshima M (2008) A hydrophilic cation-binding protein of Arabidopsis Italiana, AtPCP1, is localized to plasma membrane via N-myristoylation and interacts with calmodulin and the phosphatidylinositol phosphates PtdIns(3,4,5)P(3) and PtdIns(3,5)P(2). FEBS Lett 275: 2267–2282

Nebenführ A, Ritzenthaler C, Robinson DG (2002) Brefeldin A: deciphering an enigmatic inhibitor of secretion. Plant Physiol 130: 1102–1108

Nelson RS, Citovsky V (2005) Plant viruses: invaders of cells and pirates of cellular pathways. Plant Physiol 138: 1809–1814

Nicolas O, Dunnington SW, Gotow LF, Piron TF, Hellmann GM (1997) Variations in the VpG protein allow a potyvirus to overcome via gene resistance in tobacco. Virology 237: 452–459

Olesińska AA, Almon E, Navot N, Perl A, Galun E, Lucas WJ, Wolf S (1996) Tissue-specific expression of the tobacco mosaic virus movement protein in transgenic potato plants alters plasmodesmal function and carbohydrate partitioning. Plant Physiol 111: 541–550

Opara KJ (2004) Getting the message across: how do plant cells exchange macromolecular complexes? Trends Plant Sci 9: 33–41

Pallas V, García JA (2011) How do plant viruses induce disease? Interactions and interference with host components. J Gen Virol 92: 2691–2705

Peremysov VV, Prokhnevsky AJ, Avisar D, Don MV (2008) Two class XI myosins function in organelle trafficking and root hair development in Arabidopsis. Plant Physiol 146: 1109–1116

Perraki A, Cacas JL, Crowet JM, Lins L, Castroviejo M, German-Retana S, Mongrand S, Raffaele S (2012) Plasma membrane localization of Sola-num tuberosum remorin from group 1, homolog 3 is mediated by conformational changes in a novel C-terminal anchor and required for the restriction of potato virus X infection. Plant Physiol 166: 624–637

Pita JS, de Miranda JR, Schneider WL, Roossinck MJ (2007) Environment determines fidelity for an RNA virus replicase. J Virol 81: 9072–9077

Pouwels J, Van Der Krogt GN, Van Lent J, Bisseling T, Wellink J (2002) The cytoskeleton and the secretory pathway are not involved in

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