Dissecting the role of a plant-specific Rab5 small GTPase NbRabF1 in Bamboo mosaic virus infection

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Highlight

The cell-to-cell movement of Bamboo mosaic virus could be trafficking through the small GTPase RabF1-mediated vesicle transport from the Golgi apparatus to the plasma membrane.
Abstract

NbRabF1, a small GTPase from *Nicotiana benthamiana* and a homolog of *Arabidopsis thaliana* Ara6, plays a key role in regulating the *Bamboo mosaic virus* (BaMV) movement by transporting between endosomal membranes. Reducing the expression of NbRabF1 in *N. benthamiana* by virus-induced gene silencing decreased the accumulation of BaMV and with smaller infection foci on inoculated leaves but no effect in protoplasts. Furthermore, transient expression of NbRabF1 increased the accumulation of BaMV in inoculated leaves. Thus, NbRabF1 may be involved in the cell-to-cell movement of BaMV. The potential acyl modification sites at the second and third positions of NbRabF1 were crucial for membrane targeting and BaMV accumulation. The localization of NbRabF1 mutants with the GDP-bound (donor site) and GTP-bound (acceptor site) forms suggested that NbRabF1 might regulate vesicle trafficking between the Golgi apparatus and plasma membrane. Furthermore, GTPase activity could also be involved in BaMV cell-to-cell movement. Overall of this study, we identified a small GTPase, NbRabF1, from *N. benthamiana* that interacts with its activation protein NbRabGAP1 and regulates vesicle transport from the Golgi apparatus to the plasma membrane. The BaMV movement complex might move from cell to cell through this vesicle trafficking route.

Keywords: BaMV; cell-to-cell movement; Rab small GTPase; intracellular trafficking; *Nicotiana benthamiana*
Introduction

Rab small GTPases (Rabs) play major roles in regulating communication between organelles via vesicular transport, which allows for exchanging lipids and membrane proteins among organelles (Goody et al., 2017; Langemeyer et al., 2018). Rabs are cycled between the active (GTP-bound) and inactive (GDP-bound) forms, with GTP hydrolysis facilitated by GTPase-activating proteins (GAPs) and GDP replaced by GTP under the effect of the guanine nucleotide exchange factor (Pfeffer, 2013). In plant cells, the endocytic and vacuolar transport pathways different from non-plant cells, some of the Rabs are unique (Ueda et al., 2001).

Ara6 (RABF1) is a plant-specific Rab that is most similar to Ara7 (RABF2b), a conventional RabF2 (Rab5 in the animal system) ortholog in Arabidopsis. Ara6 contains an extra stretch of amino acids at the N-terminus harboring the alkylating residues that target the membrane, whereas with conventional RabF2 molecules such as Ara7, the membrane targeting signaling resides at the C-terminus. Both Ara6 and Ara7 localize on early endosomes, but Ara6 is also observed on the plasma membrane (Ueda et al., 2001). Ara6 regulates vesicle trafficking between endosomes and the plasma membrane (Ebine et al., 2011).

Rabs are involved in animal pathogen infection including bacteria and viruses (Ireton et al., 2014; Mottola, 2014; Spano and Galan, 2018; Spearman, 2018), and different Rabs regulate various intracellular movements of animal viruses (Chambers and Takimoto, 2010; Hsiao et al., 2015; Mainou and Dermody, 2012; Mannova and Forstova, 2003). However, only a few cases have been reported in plant viruses. The movement protein of Cauliflower mosaic virus co-localized with vesicles containing AtRAB-F2b is transported in an endocytic pathway (Carluccio et al., 2014). Rab5 is involved in recruiting the phosphatidylethanolamine-enriched membrane to build up the virus replication complex during Tobacco bushy stunt virus infection (Xu and Nagy, 2016). A Rab5 ortholog in Arabidopsis, Ara7, is involved in trafficking movement proteins of Potato mop-top virus in the early endocytic pathway (Haupt et al., 2005).

Bamboo mosaic virus (BaMV) is a flexuous-rod virus belonging to the Potexvirus genus of the Alphaflexiviridae family (Lin et al., 1977). The genome of BaMV is approximately 6.4 kb with a 5′ m7GpppG structure and a 3′ poly(A) tail; the viral RNA contains five open reading frames (ORFs) (Lin et al., 1994). ORF1 encodes a 155-kDa polypeptide for viral RNA replication with 3 functional domains: the capping enzyme domain containing the GTP methyltransferase and S-adenosylmethionine-dependent guanylyltransferase activities (Huang et al., 2004; Li et al., 2001a; Li et al., 2001b); the helicase-like domain containing the nucleoside triphosphatase and RNA 5′-triphosphatase activities (Li et al., 2001b); and the polymerase domain containing RNA-dependent RNA polymerase (RdRp) activity (Li et al., 1998; Li et al., 2001b). ORFs 2-4 encode 3 overlapped
movement proteins (termed triple gene block protein, TGBp) for viral movement (Chen et al., 2012; Lin et al., 2004; Lin et al., 2006). ORF5 encodes viral capsid protein required for viral RNA encapsidation, movement and symptom development (Hung et al., 2014a; Hung et al., 2014b; Lan et al., 2010; Lee et al., 2011).

Previously, we identified a Rab GTPase activation protein 1 from *Nicotiana benthamiana* (NbRabGAP1) involved in BaMV cell-to-cell movement (Huang et al., 2013). The result suggested that NbRabGAP1 might regulate a Rab associated with vesicle trafficking assisting BaMV movement. BaMV movement could involve shuttling between the plasma membrane and endoplasmic reticulum (ER) or early endosome (Chen et al., 2017). Therefore, the possible candidate to interact with NbRabGAP1 would be a RabF1, a Rab5 ortholog. The main goal of this study was to identify which Rab is involved in regulating BaMV movement. Here, we identified one of the *N. benthamiana* RabF1 proteins involved in BaMV movement and found the membrane-targeting and GTPase activity of this RabF1 protein critical for BaMV intercellular movement.

**Materials and methods**

**Plants and viruses**

*Nicotiana benthamiana* plants were grown in a growth chamber with 16 h light/8 h dark at 28°C described previously (Cheng et al., 2010). Virion or viral RNA of the BaMV severe strain (BaMV-S) (Lin et al., 1994) was used for inoculation.

**Constructs**

The TRV-knockdown system was described elsewhere (Huang et al., 2016). In brief, the fragment from nt 379 to 594 of *NbRabF1* was used for virus-induced gene silencing (VIGS). The fragment from nt 1 to 233 was used to determine the silencing efficiency. For transient expression, the cDNA of NbRabF1 was amplified by using the primers BamHI/NbRabF1/F (5’-GGGGATCCATGGGTTGCGCATCTTCAGTTG-3’) and KpnI/NbRabF1/R (5’-GGGGGTACCAGCAGCAGACGGGCG-3’) and cloned into the pGEM-T Easy vector (Promega). The resulting clone was digested with *BamH*1 and *Kpn*1 and cloned into pBin-mGFP with GFP fused at the C-terminus of NbRabF1. For the membrane-targeting mutants, the forward primers BamHI/NbRabF1G2A/F (5’-GGGGGATCCATGGCTTGCGCATCTTCAGTTG-3’), BamHI/NbRabF1C3A/F (5’-GGGGGTACCAGGCAGCAGACGGGCG-3’) and BamHI/NbRabF1G2AC3A/F (5’-GGGGGATCCATGGCTGCCGCATCTTCAGTTG-3’) containing
the mutation sequences were used to create G2A, C3A and G2AC3A mutants, respectively. To clone the GTP- and GDP-bound mutants, Q92L and S46N, BamHI/NbRabF1/F and NbRabF1/Q92L/R (5′-GCGTACCTCTCCAGACCCG-3′) or NbRabF1/S46N/R (5′-GCAAAACAATACAGTTTTTACCAAC-3′), respectively, were used for PCR to generate mega-primers. The mega-primers were gel-eluted and used with KpnI/NbRabF1/R for second PCR.

**VIGS and virus inoculation**

The TRV-based silencing system used for VIGS was described previously (Huang et al., 2016). In short, the NbRabF1 fragment was inserted into a TRV2-containing vector and transformed into agrobacteria. TRV1- and TRV2-harboring agrobacteria were mixed at a 1:1 ratio and infiltrated into second to fourth true leaves of *N. benthamiana* at the five-leaf stage. Eighth and ninth true leaves were inoculated with 100 ng BaMV viral particle and harvested at 5 days post-inoculation (dpi). The inoculated leaves were harvested from each individual plant for further analysis, and at least three plants were analyzed for each treatment.

**Measurement of GFP foci**

As mentioned in the previous section, eighth and ninth true leaves at 12 days post-agro-infiltration were inoculated with 100 ng BaMV-GFP viral particles isolated from pCBG-inoculated leaves (Huang et al., 2016) and harvested at 5 dpi. The GFP foci on inoculated leaves were detected by fluorescent microscopy with excitation wavelength 488 nm. The size of each focus was measured by using the software CellSens (Olympus, Japan).

**Knockdown-protoplast isolation and viral RNA inoculation**

As mentioned previously, eighth and ninth true leaves from knockdown plants were harvested for protoplast isolation at 14 days post-agro-infiltration. Approximately 1 μg BaMV viral RNA was inoculated into isolated protoplasts as described (Huang et al., 2016). Total protein was extracted at 24 hr post-inoculation. Western blot analysis was used to determine the accumulation of BaMV coat protein (CP).
**Transient expression of NbRabF1 and its derivatives**

In general, agrobacteria containing NbRabF1 or its derivatives were mixed with agrobacteria harboring the silencing suppressor HcPro at a 1:1 ratio and infiltrated onto the third to fifth leaves at the six-leaf stage. One day after infiltration, 100 ng BaMV virion was inoculated into infiltrated leaves and harvested at 5 dpi. The expression protocol was previously described (Cheng et al., 2013).

**Fractionation of NbRabF1 and its derivatives**

NbRabF1 and its derivatives (NbRabF1/G2A, -/C3A or -/G2AC3A) were transiently expressed by agro-infiltration into *N. benthamiana* leaves at the seven-leaf stage. Approximately 0.5 g infiltrated-leaf was collected at 3 days post infiltration. Total protein was homogenized (RetschTM MM400 Mixer Mill) with 1 ml pre-chilled buffer A (10 mM sodium phosphate, pH 7.4, 100 mM NaCl) containing 2 mM β-mercaptoethanol and 1X protease inhibitor cocktail (Roche, USA). The samples were centrifuged at 12000×g for 5 min at 4°C. The supernatant was used as cytosol fraction. The pellet was washed twice with 1 ml buffer A and centrifuged at 12000×g for 5 min at 4°C to remove the washing solution. After washing, the pellet was stirred gently with 1 ml buffer A containing 2% Triton X-100 for 30 min at 4°C, then centrifuged at 12000×g for 5 min at 4°C. The supernatant was collected as a membrane fraction. Western blot analysis was used to quantify the amount of each fraction of NbRabF1 and its derivatives (Cheng et al., 2013).

**Western blot analysis**

Total protein was extracted from BaMV-inoculated leaves with extraction buffer (50 mM Tris-HCl pH 8.0, 10 mM KCl, 10 mM MgCl2, 1 mM EDTA, 20% glycerol, 2% SDS, 10% β-mercaptoethanol) and separated by 12%-SDS PAGE. The BaMV CP was detected by using rabbit anti-BaMV CP polyclonal antibodies. Rabbit anti-GFP antibody was used to detect GFP-fused NbRabF1 and its derivatives. Rabbit anti-actin was used to detect endogenous β-actin for normalization. Fluorescent-labeled anti-rabbit IgG (Rockland Immunochemicals Inc., USA) was a secondary antibody. The fluorescent signal on the membrane was visualized and quantified by using LI-COR Odyssey (LI-COR Biosciences, USA). The RuBisCo large subunit (rbcL) was stained with Coomassie brilliant blue for normalization in the transient expression experiment.
Subcellular localization by laser scanning confocal microscopy

Agrobacteria containing pBIN/AtARA6-OFP, -/NbRabF1-GFP, -/NbRabF1S46N-GFP, -/NbRabF1Q92L-GFP, -/NbTRXh2-OFP (plasma membrane marker) (Chen et al., 2017), pCD3-967 (Golgi mCherry marker), pCD3-975 (tonoplast marker) (Nelson et al., 2007) and pBIN61/HcPro were cultured and induced with 450 μM acetosyringone in 10 mM MgCl₂ to a final optical density of 600 nm (OD600) = 1. Agrobacteria harboring the pBIN/NbRabF1-GFP and its derivatives were mixed pairwise with the above organellar markers and that containing pBIN61-HcPro in a 1:1:1 ratio. All mixtures were infiltrated into N. benthamiana leaves for 3 days, and protoplasts were isolated from infiltrated leaves. Images were obtained by using an Olympus Fluoview FV1000 laser scanning confocal microscope with 488- and 543-nm laser excitation for GFP and mCherry, respectively, and 515-nm excitation for YFP and OFP.

Immunoprecipitation assay

Agrobacteria containing pBIN/YFP, -/AtARA6-YFP, -/YFP-AtARA7, -/GFP, -/NbRabF1-GFP or -/NbRabF1/Q92L-GFP were mixed with that containing pBIN61/HcPro and -/NbRabGAP1-T7 in a 1:1:1 ratio, respectively, and infiltrated into N. benthamiana leaves for 3 days. The GFP/YFP-comprising proteins from transiently expressed leaves were extracted with the buffer 20 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 300 mM NaCl, 5 mM DTT, 2.5% PVPP and 1X protease inhibitor (Roche) and purified by using the GFP-Trap agarose kit (Chromotek, Martinsried, Germany). The pull-down proteins were analyzed by western blot analysis.

Yeast two-hybrid assay

The gene fragments of NbRabGAP1 or the interacting domain (TBC) NbRabGAP1/TBC and NbRabF1 or GTP-bound mutant NbRabF1/Q92L were cloned into the prey plasmid pYESTrp and bait plasmid pHyLex/Zeo, respectively (Invitrogen, Carlsbad, CA, USA). The proteins of NbRabGAP1 or NbRabGAP1/TBC and NbRabF1 or NbRabF1/Q92L were co-expressed in Saccharomyces cerevisiae strain L40, respectively, and selected on Trp-/Zeocin plates. The interaction was tested on Trp-/His-/Zeocin plates when pYESTrp-Jun and pHyLEX/Zeo-Fos was as the positive control and pYESTrp-Jun and pHyLEX/Zeo-Lamin as the negative control.
Expression and purification of His-tagged proteins from E. coli

The coding sequence of \( \text{NbRabF1} \) was amplified with the primer set KpnI-5'-1 RabF1 (5'-GGTACCATGGTGATCGCATCTTCAGT-3') and XhoI-3'-RabF1 (5'-GCTCGAGAGCAGACGGGCGTGTTAAG-3') (KpnI and XhoI site underlined, respectively). The PCR product was cloned into the pGEM-T easy vector and verified by sequencing. Finally, \( \text{NbRabF1} \) was subcloned from the T-vector into the pET29a (+) expression vector (Invitrogen) and transformed into \( E. \ coli \) BL21(DE3). The resulting clone was designated pET29a (+)-\( \text{NbRabF1} \).

\( E. \ coli \) containing pET29a(+)-\( \text{NbRabF1} \) was cultured to OD\textsubscript{600} = 1 (120 ml in total volume), the expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside at 16°C for 1 d, then samples were centrifuged at 7000 rpm at 4°C for 7 min. The cell pellet was resuspended with 8 ml buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 2 mM DTT) containing protease inhibitor cocktail (Roche, Germany) and subjected to the sonifier (Sonicator 3000, Misonix) at 4°C with 15% amplitude at 10-sec intervals for 20 min. The cell extract was clarified by centrifugation at 12,000 rpm for 5 min then incubated with 1 ml High-Capacity Profinity™ IMAC Resins (Bio-Rad, California, USA) at 4°C for 1 hr, washed with 10 ml buffer A containing 25 mM imidazole, and eluted with buffer A containing 250 mM imidazole. Finally, the eluted protein was passed through Sephacryl S-300 gel filtration at a flow rate of 1 ml/min and dialyzed with buffer containing 20 mM Tris-HCl pH 8.0, 50 mM NaCl, and 10% glycerol. The control His-GFP construct was manipulated under the same condition.

GTPase activity

The GTPase activity of \( \text{NbRabF1} \) was analyzed with Transcreener® GDP FI assay (BellBrookLabs, Madison, WI, USA) described previously (Hoepflinger et al., 2013). The purified recombinant protein of \( \text{NbRabF1} \) GTPase was applied in the assay. When the reaction product GDP were released and displaced Alexa Fluor 594-GDP tracer bound to a GDP antibody–IR dye quencher conjugate, the signal of Alexa Fluor 594 can be detected and quantified by SpectraMax M2 (Molecular Devices, USA). The excitation wavelength is 584 nm and the emission wavelength is 610 nm. The recombinant proteins were diluted with the buffer containing 50 mM HEPES (pH 7.5), 4 mM MgCl2, 2 mM EGTA, 1% DMSO, and 0.01% Triton X-100. Approximately 1.5 μM recombinant protein was applied to assay with 10 μM GTP or without GTP. All measurements were performed in three replicates at room temperature according to manufacturer’s instruction.
**BiFC assay**

To construct NbRabF1 for BiFC assay, GFP and OFP sequence on pBIN-NbRabGAP1-OFP, pBIN-NbRabF1-GFP and pBIN-NbRabF1Q92L-GFP was replaced by nYFP and cYFP sequence, respectively. The primer set for cloning nYFP and cYFP was KpnI-nYFP (5'-GGTACCATGGTGAGCAA-3')/SacI-nYFP (5'-GAGCTCTCAGTGATGT-3') and KpnI-cYFP (5'-GGTACCATTGGGCAGCGTGCA-3')/SacI-cYFP (5'-GAGCTCTCAGTGATGT-3'), respectively. Agrobacteria containing pBIN-NbRabGAP1-nYFP was mixed with that containing pBIN-NbRabF1-cYFP or pBIN-NbRabF1Q92L-cYFP in a 1:1 ratio and infiltrated onto N. benthamiana leaves for 3 d, while the pBIN-nYFP/-cYFP, pBIN-NbRabGAP1-nYFP/-cYFP, pBIN-nYFP/-NbRabf1-cYFP, and pBIN-nYFP/-NbRabf1Q92L-cYFP were negative controls. On the other hand, Agrobacteria containing pBIN-NbRabF1-nYFP or pBIN-NbRabF1Q92L-nYFP was mixed with that containing pBIN-NbRabGAP1-cYFP in a 1:1 ratio to confirm the interaction between NbRabGAP1 and NbRabF1. Images were obtained with an Olympus Fluoview 3000 laser scanning confocal microscope using 514 nm laser excitation for YFP.

**Results**

*Two RabF1 isoforms were identified in N. benthamiana*

Results from previous study indicated that BaMV movement was regulated by a Rab-GTPase-activating protein, NbRabGAP1 (Huang et al., 2013), and might be trafficked from the ER membrane to plasma membrane (Chen et al., 2017). The possible Rab candidate involved in vesicle trafficking in this route is RABF (Ebine et al., 2011; Ueda et al., 2001).

To search for the member of RABF family is the target of NbRabGAP1 from N. benthamiana could be involved in BaMV infection, the plant-specific RabF1 sequence from Arabidopsis AtAra6 (accession no.: AB007766) (Altschul et al., 1997) was used to compared the N. benthamiana database (http://benthgenome.qut.edu.au/). Two sequences, NbRab6a and NbRab6b, with only one amino acid difference were obtained (Fernandez-Pozo et al., 2015) and aligned with that of AtAra6 (Fig. 1A). On sequence alignment, NbRab6a and AtAra6 showed 87% identity. We cloned the coding region of the two genes, NbRab6a and NbRab6b, and expressed it in N. benthamiana cells. On subcellular localization analysis, NbRab6a-GFP and NbRab6b-OFP co-localized (Fig. 1B). Thus, the two isoforms could be functionally identical. The sequence of NbRab6a was then used to represent NbRabF1 for further analysis.
NbRabF1 assists BaMV infection

To investigate the effect of NbRabF1 on BaMV infection, we used Tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) to knock down the expression of NbRabF1. Because the region chosen for knocking down the expression of NbRabF1 is the same sequence between Rab6a and Rab6b, both genes if they all expressed would be knocked down with VIGS. The phenotype of NbRabF1-knockdown plants did not differ from that of control Luc-knockdown plants (Fig. S1). The expression of NbRabF1 was reduced to 18% of that of the control (Luc-knockdown leaves) (Fig. 2A). The accumulation of BaMV coat protein (CP) in NbRabF1-knockdown leaves was reduced to ~77% (Fig. 2B), with no significant effect on BaMV accumulation in NbRabF1-knockdown protoplasts (Fig. 2C). Thus, NbRabF1 was likely involved in BaMV cell-to-cell movement. To validate this hypothesis, we used fluorescent microscopy to measure the size of BaMV infection foci on the NbRabF1-knockdown leaves. Because the infectious BaMV cDNA clone was constructed to express GFP, the infection foci represented by the green fluorescence could be directly measured (Fig. 3A). The mean size of BaMV infection foci was significantly smaller on NbRabF1-knockdown than Luc-knockdown leaves (Fig. 3B).

On transient expression of NbRabF1 in N. benthamiana leaves followed by BaMV inoculation, BaMV accumulation was significantly increased to 137% of that of the control at 5 dpi (Fig. 4). Together, the results from loss-of-function (knockdown experiments) (Fig. 3) and gain-of-function (transient expression experiments) (Fig. 4) indicated that NbRabF1 facilitated BaMV intercellular movement.

The membrane-targeting of NbRabF1 is required for BaMV accumulation

The sequence analysis and comparison of NbRabF1 with other RABF1 members indicated that the glycine at the second amino acid (G2) and cysteine at the third amino acid (C3) are conserved (Fig. 1A). The G2 and C3 were found to be N-myristoylated and palmitoylated, respectively, for membrane targeting (Ueda et al., 2001). To demonstrate that the membrane targeting of NbRabF1 is crucial for BaMV accumulation, we substituted the amino acid G2 and C3 for alanine to produce NbRabF1/G2A, -/C3A and -/G2AC3A. Fractionation analysis indicated that NbRabF1 was mainly localized at the membrane fraction (Fig. 5A). By contrast, most of NbRabF1/G2A was localized at the cytosol. However, approximately half of NbRabF1/C3A remained in the membrane fraction (Fig. 5A). Thus, G2 myristoylation may be more critical than C3 palmitoylation for NbRabF1 to target the membrane. The double mutant NbRabF1/G2AC3A mostly distributed to the cytosol in the fractionation assay. Confocal data also confirmed these results: G2A was distributed in the cytoplasm and most of C3A remained in the membrane, whereas G2AC3A was localized to both cytoplasm and nucleus (Fig. 5B).
The results suggested that G2-myristoylation and C3-palmitoylation are the only two amino acid functions involved in targeting the membrane.

The expression of NbRabF1/G2AC3A-GFP showed reduced ability to assist BaMV accumulation as compared with the wild type (Fig. 4). Thus, membrane-targeting of NbRabF1 is critical for BaMV accumulation. The results also implied that the BaMV movement complex could be trafficked through the vesicle with the assistance of NbRabF1.

**NbRabF1 regulates vesicle trafficking from the Golgi apparatus to plasma membrane**

To reveal the intracellular vesicle trafficking pathway of NbRabF1, we used a few known organellar markers that co-localize with NbRabF1. NbRabF1-GFP co-localized with Golgi (Man49)-mCherry and the endosomal marker AtAra6-OFP (Haas et al., 2007; Ueda et al., 2001) (Fig. 6). Based on the structural analysis in Rab proteins (Dumas et al., 1999; Lamers et al., 2017; Lee et al., 2009), the β1/α1 loop provides phosphate contacts and a Ser/Thr residue (correspond to the S46 of NbRabF1) to coordinate the Mg^{2+} ion. Mutant S46N could disrupt the binding of Mg^{2+} molecule essential for GTP binding and consequently locking the GTPase in an inactive form (GDP-bound). By contrast, mutant Q92L could affect the conserved residue critical for catalysis and consequently fix the GTPase in a GTP-bound form. Therefore, to determine the donor and acceptor compartments of the vesicle associated with NbRabF1, we constructed mutant NbRabF1/S46N (GDP-bound form) and NbRabF1/Q92L (GTP-bound form) fused with GFP according to the structural analysis. NbRabF1/S46N representing the donor compartment co-localized with the Golgi marker; and NbRabF1/Q92L representing the acceptor compartment co-localized with the plasma membrane marker, NbTRXh2-OFP (Chen et al., 2018) (Fig. 6). From the confocal images, we proposed that NbRabF1 could likely shuttle between the Golgi apparatus and plasma membrane through the endosomal system.

**GTPase activity of NbRabF1 might be critical for efficient BaMV infection**

To examine whether the NbRabF1 we cloned harbors the GTPase activity, we overexpressed and purified the protein from *E. coli*. The GTPase activity was then tested with labeled-GDP fluorescence intensity readout assay (Hoepflinger et al., 2013). The result indicated that NbRabF1 has the relative GTPase activity higher than that of the control (the GFP-only expressed and purified from *E. coli*) (Fig. S2). We then expressed NbRabF1/S46N (GDP-bound form at Golgi) and NbRabF1/Q92L (GTP-bound form at plasma membrane) in leaves, followed by BaMV inoculation. The expression of NbRabF1/S46N had no effect on BaMV
accumulation. By contrast, the expression of NbRabF1/Q92L had a negative effect on BaMV accumulation (Fig. 4). These results suggest that the GTPase activity of NbRabF1 might be playing a critical role in assisting BaMV movement. Mutant NbRabF1/Q92L with the stabilized GTP-bound form and the GTP failing to be hydrolyzed could have a negative effect on BaMV accumulation.

**NbRabF1 interacts with NbRabGAP1**

To examine whether NbRabGAP1 targets NbRabF1, we co-expressed NbRabGAP1-OFP and NbRabF1/Q92L-GFP in *N. benthamiana* cells. The two proteins co-localized on the plasma membrane (Fig. 7A). To validate the interaction between NbRabGAP1 and NbRabF1, we used co-immunoprecipitation assay (Fig. 7B) and yeast two-hybrid experiment (Fig. 7C). NbRabGAP1-T7 could be detected only when co-expressed with NbRabF1-GFP or NbRabF1/Q92L-GFP but not GFP alone in *N. benthamiana* cells (Fig. 7B). The results of yeast two-hybrid experiment confirmed the interaction of NbRabGAP1 with NbRabF1 or NbRabF1/Q92L. The interaction through the TBC domain of NbRabGAP1 was also confirmed (Fig. 7C). Hence, BaMV may move in *N. benthamiana* cells via vesicle trafficking regulated by NbRabF1, activated by NbRabGAP1 on the plasma membrane.

Furthermore, we also used bimolecular fluorescence complementation assay to visualize the interaction in live cells. The fluorescent signal could only be detected when NbRabGAP1-nYFP was co-expressed with NbRabF1-cYFP or NbRabF1/Q92L-cYFP or reverse constructs (Fig. 8).

**Discussion**

Rabs are a group of small GTPases that regulate vesicle formation through which materials such as cargo can be transported between organelles. In this study, we identified one Rab, NbRabF1, involved in BaMV movement. The subcellular localization of NbRabF1 is similar to that of CaAra6 from green algae, which localizes to the Golgi apparatus, endosomes and plasma membrane (Hoepflinger et al., 2013). However, unlike AtAra6 (Ueda et al., 2001), NbRabF1 also co-localized with the Golgi (TGN) marker, which was further identified to be the location of the GDP-bound form NbRabF1/S46N indicated as a donor site of vesicle formation. The GTP-bound form NbRabF1/Q92L co-localized with the plasma membrane marker indicated as the recipient site of the transport (Fig. 5). These results suggest that NbRabF1-containing vesicles bud out from the Golgi apparatus and travel to the plasma membrane through the endosomal pathway. The BaMV viral movement complex might take
advantage of this novel mechanism to traffic from the Golgi apparatus to plasma membrane and then move toward plasmodesmata.

Expression of the GDP-bound form NbRabF1/S46N had no effect on BaMV accumulation (Fig. 4). By contrast, the other mutant with a GTP-bound form, NbRabF1/Q92L, had a dominant negative effect when expressed. These results suggest that the NbRabF1/Q92L with a GTP-bound form might interact with the GAP protein and be stocked on the plasma membrane. This situation could have an effect on recycling the endogenous NbRabF1 required for BaMV movement. However, this dominant negative effect was not observed on the other side of the trafficking pathway: NbRabF1/S46N with the GDP-bound form and stocked on the donor membrane (Golgi), had no effect on BaMV trafficking (Fig. 4). The endogenous NbRabF1 may be mostly on endosomal and Golgi membranes (Fig. 5) and that recharging of the inactive form (GDP bound) to the active form (GTP bound) of NbRabF1 is not a critical step in transporting the cargos (including BaMV) to the plasma membrane. Therefore, the effect of retarding transportation was not observed when NbRabF1/S46N was expressed and trapped at the Golgi membrane (Fig. 4).

The assembly of the BaMV movement complex is believed to be initiated at the ER membrane, where the two transmembrane viral movement proteins, TGBp2 and TGBp3 (Chou et al., 2013) are translated. The two movement proteins are recruited to assemble the viral movement complex containing viral RNA, TGBp1, replicase, and CP at the ER or Golgi membrane. The viral movement complex then moves to the plasma membrane via the endosomal system by vesicle trafficking with the activation of NbRabF1. Although the details of viral movement complex trafficking to the plasma membrane is not clear, the host factor elicitor-inducible leucine-rich repeat receptor-like protein (NbEILP) is involved (Chen et al., 2017). NbEILP could carry a signal to be recruited to the NbRabF1-induced vesicle, and one of the components in the viral movement complex possibly interacts with NbEILP. Therefore, the BaMV movement complex could hitch-hike the trafficking vesicle from the Golgi apparatus to the plasma membrane and then reach plasmodesmata. However, the viral complex on the plasma membrane could be negatively regulated by two host factors, the plasma membrane-associated cation-binding protein 1-like protein (Huang et al., 2017) and a thioredoxin, NbTRXh2 (Chen et al., 2018). Via the interaction of these two host proteins with the replicase in the viral movement complex, the cell-to-cell movement is retarded.

This project was initiated to search for a Rab together with its partner, NbRabGAP1 (Huang et al., 2013), in assisting BaMV cell-to-cell movement. In the co-immunoprecipitation assay, we revealed the physical interaction of the Ara6 homolog, NbRabF1, from N. benthamiana with NbRabGAP1 (Fig. 7). Two Rabs, NbRabG3f (Huang et al., 2016) and NbRabF1 (this study), and one GAP, NbRabGAP1 (Huang et al., 2013), participate in BaMV infection. These results suggested that the two Rabs localized on the Golgi apparatus and regulated the vesicle trafficking. NbRabG3f mainly
participates in BaMV replication and possibly its movement as well (Huang et al., 2013), and NbRabF1 is involved in BaMV movement. Further study could reveal how these two Rabs on the same donor membrane site but regulating vesicles (possibly with different cargoes) target the different acceptor membrane.
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Author contributions

YPH, PYH, and IHC performed the experiments; YHH, CHT and CPC took part in data analysis; CHT and CPC designated the research and wrote the article.

Data availability statement

The data supporting the findings of this study are available from the corresponding author, (CHT and CPC), upon request.
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**Figure legends**

**Fig. 1.** Alignment of amino acid sequence and subcellular localization of NbRab6. (A) The sequence alignment of *Nicotiana benthamiana* NbRab6a (Niben101Scf29276g00003.1 from Sol Genomics Network) and NbRab6b (Niben101Scf00648g00003.1 from Sol Genomics Network) and *Arabidopsis thaliana* AtAra6 (GenBank: AB007766). (B) Subcellular localization of GFP-fused NbRab6a and OFP-fused NbRab6b co-expressed in *N. benthamiana* leaves. Protoplasts were isolated from leaves for confocal imaging. GFP is in green, and OFP is in red. Scale bar = 10 μm.

**Fig. 2.** Relative accumulation of BaMV coat protein (CP) in *NbRabF1*-knockdown plants and protoplasts. (A) Real-time qRT-PCR of mRNA expression of *NbRabF1* in Luc- and *NbRabF1*-knockdown leaves. The expression of *NbRabF1* in control plants (Luc) was set to 100%. (B) and (C) Western blot analysis of BaMV CP accumulation on the inoculated leaves at 5 days post-inoculation and in protoplasts at 24 hr post-inoculation, respectively. The accumulation of control plants or protoplasts was set to 100%. Data are the mean±SD relative levels from the number of independent experiments indicated as N with the number of plants indicated as n in each experiment. Representative western blot results of CP level with RuBisCO large subunit (*rbcL*) were a control (the loading control for normalization). ***P<0.001 by Student t-test.

**Fig. 3.** Cell-to-cell movement of BaMV in Luc- and *NbRabF1*-knockdown leaves. (A) The area of fluorescent foci in inoculated leaves of Luc-knockdown control (Luc) and *NbRabF1*-knockdown (NbRabF1) plants after inoculation with the BaMV infectious plasmid carrying GFP. Scale bar = 1 mm. (B) The quantification results were derived from (a). Data are mean±SD of 86 and 72 foci from Luc- and *NbRabF1*-knockdown plants, respectively. *** P<0.001 by Student t-test.

**Fig. 4.** The accumulation of BaMV in *N. benthamiana* leaves expressing NbRabF1 or its derivatives. (A) The protein expression of NbRabF1-OFP and its derivatives in *N. benthamiana* leaves with antibody against OFP was determined by western blot analysis. (B) The relative accumulation of BaMV CP was determined by western blot analysis. Total protein was extracted from BaMV-inoculated leaves transiently expressed with OFP, NbRabF1-OFP, NbRabF1/S46N-OFP, NbRabF1/Q92L or NbRabF1/G2AC3A-OFP at 5 days post-inoculation. Data are the mean±SE from three independent experiments. The expression of actin was a loading control. * P < 0.05 by Student t-test.
Fig. 5. Fractionation and subcellular localization of NbRabF1 and its targeting mutants. (A) Western blot analysis of NbRabF1-GFP and its targeting mutants -/G2A-GFP, -/C3A-GFP and -/G2AC3A-GFP in the cytoplasm (C) or membrane (M) fraction. (B) GFP only and NbRabF1-GFP and its derivatives were transiently expressed in N. benthamiana leaves. Protoplasts were isolated from these leaves and examined by confocal microscopy. GFP is shown in green and the autofluorescence of chloroplasts is in red. Scale bar = 20 µm.

Fig. 6. Subcellular localization of NbRabF1 and its derivatives with various organellar markers. The expression of GFP-fused NbRabF1 and its derivatives is in green. The organellar markers fused with OFP or mCherry are in red. Scale bar = 20 µm.

Fig. 7. Subcellular localization and the interaction of NbRabGAP1 with NbRabF1. (A) OFP-fused NbRabGAP1 was co-expressed with NbRabF1/Q92L in N. benthamiana leaves. Protoplasts were isolated from these treated leaves for confocal imaging. GFP is in green, and OFP is in red. Scale bar = 20 µm. (B) Total proteins (input) were extracted from N. benthamiana leaves and immunoblotted with antibodies against T7-tag for NbRabGAP1 as indicated and GFP for NbRabF1 and NbRabF1/Q92L. Total proteins were then immunoprecipitated with anti-GFP beads (IP/GFP) and immunoblotted (WB) with the antibody against GFP or T7-tag (bottom). (C) Interaction of NbRabF1 with NbRabGAP1 in yeast cells. Yeast strain L40 co-transformed with the indicated plasmids was subjected to 10-fold serial dilution and incubated with minimal medium lacking tryptophan and histidine supplemented with Zeocin to identify protein interactions. Yeast containing pYESTrp-Jun and pHyLEX/Zeo-Fos was a positive control; yeast containing the vector pYESTrp-Jun and pHyLEX/Zeo-Lamin was a negative control.

Fig. 8. Bimolecular fluorescence complementation (BiFC) assay. The amino-terminal fragment (nYFP) or carboxyl-terminal fragment (cYFP) of YFP were fused to the C-terminus of NbRabF1 (shown as RabF1-nYFP or RabF1-cYFP, respectively), NbRabF1/Q92L (shown as RabF1/Q92L-nYFP or RabF1/Q92L-cYFP, respectively), or NbRabGAP1 (shown as RabGAP1-nYFP or RabGAP1-cYFP, respectively). The paired coexpression of those fused with nYFP or cYFP was indicated above each panel. Images were obtained with an Olympus Fluoview 3000 laser scanning confocal microscope using 514 nm laser excitation for YFP. The DIC images were also shown under each fluorescent image.
Figure 1

A

|   | 1  | 10 | 20 | 30 | 40 | 50 | 60 |
|---|----|----|----|----|----|----|----|
| NbRab6a | MGCA5VADRNSGRAAGLN-PDGAGAVDFKNNLKVGLVLGDSVGKSCIVLRFVRQQFP |
| NbRab6b | MGCA5VADRNSGRAAGLN-PDGAGAVDFKNNLKVGLVLGDSVGKSCIVLRFVRQQFP |
| AtAra6  | MGCA5LFDERSGTLGDSNENAYTAANLKVGLVLGDSVGKSCIVLRFVRQQFP |

|   | 60 | 80 | 100 | 120 |
|---|----|----|-----|-----|
| NbRab6a | I5KVTVGASFLSQTIALQSTTVFEEWDIAQGERAAALFLLYRGA2EAVVWYDITSPE |
| NbRab6b | I5KVTVGASFLSQTIALQSTTVFEEWDIAQGERAAALFLLYRGA2EAVVWYDITSPE |
| AtAra6   | I5KVTVGASFLSQTIALQSTTVFEEWDIAQGERAAALFLLYRGA2EAVVWYDITSPE |

|   | 120 | 140 | 150 | 170 | 180 |
|---|-----|-----|-----|-----|-----|
| NbRab6a | FFAAQYVWKEQGKSFDIVMALVGHRADLHEKREVTPQGGIDCAGKNGFFIETSATK |
| NbRab6b | FFAAQYVWKEQGKSFDIVMALVGHRADLHEKREVTPQGGIDCAGKNGFFIETSATK |
| AtAra6   | FFAAQYVWKEQGKSFDIVMALVGHRADLHEKREVTPQGGIDCAGKNGFFIETSATK |

|   | 180 | 190 | 202 |
|---|-----|-----|-----|
| NbRab6a | ADHINQLPEEIAKRLPFRSAAA- |
| NbRab6b | ADHINQLPEEIAKRLPFRSAAA- |
| AtAra6   | ADHINQLPFRIAKRLPFRSAAA- |

B

|   | NbRab6a-GFP | NbRab6b-OFP | Merge |
|---|-------------|-------------|-------|
| 16 | [Image of green fluorescence] | [Image of red fluorescence] | [Image of merged fluorescence] |
| 10 | [Image of green fluorescence] | [Image of red fluorescence] | [Image of merged fluorescence] |
| 10 | [Image of green fluorescence] | [Image of red fluorescence] | [Image of merged fluorescence] |

[Caption: Figure 1 shows the amino acid sequences for NbRab6a, NbRab6b, and AtAra6. Part A displays the sequences for the 60th to 180th amino acids. Part B illustrates the fluorescence images for NbRab6a-GFP, NbRab6b-OFP, and their merged image.]
Figure 4

(A) Molecular mass markers (kDa). 

(B) The relative accumulation of Rab11a coat proteins (%). 

| Condition          | Relative Accumulation | p value |
|--------------------|-----------------------|---------|
| GFP -GFP           | 100                   | 0.034   |
| NbRabF1 -GFP       | 137 ± 17              | 0.74    |
| /S46N -GFP         | 97 ± 14               | 0.011   |
| /Q92L -GFP         | 63 ± 15               | 0.016   |
| /G24C3A -GFP       | 802.21                | 0.16    |

CP and actin are shown as controls.
Figure 5

|                   | 
|-------------------|
| **A**             | 
| **B**             |

| NbRabF1-GFP       | NbRabF1/G2A-GFP | NbRabF1/C3A-GFP | NbRabF1/G2AC3A-GFP |
|-------------------|-----------------|-----------------|--------------------|
| C                 | M               | C               | M                  |
|                   |                 |                 |                    |

B

| **GFP**          | **autofluorescent** | **Merge** | **Bright field** |
|------------------|--------------------|-----------|------------------|
| **GFP only**     |                    |           |                  |
| **NbRabF1-GFP**  |                    |           |                  |
| **NbRabF1/G2A-GFP** |                  |           |                  |
| **NbRabF1/C3A-GFP** |                 |           |                  |
| **NbRabF1/G2AC3A-GFP** |              |           |                  |
Figure 6

GFP          OFP/mCherry   Merge        Bright field

NbRabF1-GFP  
Golgi-mCherry
(Golgi marker)

NbRabF1-GFP  
AtARA6-OFP
(endosomal marker)

NbRabF1/Q92L-GFP  
NbTRXh2-OFP
(plasma membrane
marker)

NbRabF1/S64N-GFP  
Golgi-mCherry
(Golgi marker)
Figure 7

A

GFP only
NbRabGAP1
-OFP

NbRabF1/Q92L
-GFP
NbRabGAP1
-OFP

B

|           | GFP | +  | -  | -  |
|-----------|-----|----|----|----|
| NbRabF1-GFP | -   | +  | -  | -  |
| NbRabF1/Q92L-GFP | -   | -  | +  | +  |
| NbRabGAP1-T7 | +   | +  | +  | +  |

input

WB/GFP

GFP

WB/T7

NbRabGAP1-T7

WB/actin

IP/GFP

WB/GFP

NbRabF1-GFP

NbRabF1/Q92L-GFP

GFP

WB/T7

NbRabGAP1-T7

C

| Trp"/Zeocin | positive | negative |
|-------------|----------|----------|
| 1           |          |          |
| 10^{-1}     |          |          |
| 10^{-2}     |          |          |
| 10^{-3}     |          |          |

| Trp"/His"/Zeocin |          |          |
|------------------|----------|----------|
| 1                |          |          |
| 10^{-1}          |          |          |
| 10^{-2}          |          |          |
| 10^{-3}          |          |          |
Figure 8

nYFP  nYFP  nYFP  RabGAP1-nYFP
  cYFP  RabF1-cYFP  RabF1/Q92L-cYFP  RabGAP1-cYFP

RabGAP1-nYFP  RabGAP1-nYFP  RabF1-nYFP  RabF1/Q92L-nYFP
  RabF1-cYFP  RabF1/Q92L-cYFP  RabGAP1-cYFP  RabGAP1-cYFP

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