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NbRABG3f, a member of Rab GTPase, is involved in *Bamboo mosaic virus* infection in *Nicotiana benthamiana*

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SUMMARY

The screening of differentially expressed genes in plants after pathogen infection can uncover the potential host factors required for the pathogens. In this study, an up-regulated gene was identified and cloned from *Nicotiana benthamiana* plants after *Bamboo mosaic virus* (BaMV) inoculation. The up-regulated gene was identified as a member of the Rab small guanosine triphosphatase (GTPase) family, and was designated as *NbRABG3f* according to its *in silico* translated product with high identity to that of *RABG3f* of tomato. Knocking down the expression of *NbRABG3f* using a virus-induced gene silencing technique in a protoplast inoculation assay significantly reduced the accumulation of BaMV. A transiently expressed NbRABG3f protein in *N. benthamiana* plants followed by BaMV inoculation enhanced the accumulation of BaMV to approximately 150%. Mutants that had the catalytic site mutation (NbRABG3f/T22N) or had lost their membrane-targeting capability (NbRABG3f/ΔC3) failed to facilitate the accumulation of BaMV in plants. Because the Rab GTPase is responsible for vesicle trafficking between organelles, a mutant with a fixed guanosine diphosphate form was used to identify the donor compartment. The use of green fluorescent protein (GFP) fusion revealed that GFP-NbRABG3f/T22N clearly co-localized with the Golgi marker. In conclusion, BaMV may use NbRABG3f to form vesicles derived from the Golgi membrane for intracellular trafficking to deliver unidentified factors to its replication site; thus, both GTPase activity and membrane-targeting ability are crucial for BaMV accumulation at the cell level.

Keywords: *Bamboo mosaic virus*, Golgi membrane, Rab GTPase, vesicle transport.

INTRODUCTION

The Rab guanosine triphosphatase (GTPase) family is part of the small GTPase superfamily that regulates the intracellular transport of vesicles. The switching of guanosine-5'-triphosphate (GTP)-bound active and guanosine diphosphate (GDP)-bound inactive forms is regulated by GTPase-activating proteins (GAPs), which hydrolyse GTP, and the guanine nucleotide exchange factor (GEF), which catalyses GTP binding (Pfeffer, 2013; Saito and Ueda, 2009; Stenmark, 2009). Each step in the vesicular transport pathway requires at least one Rab. Each type of Rab exhibits a characteristic distribution on organelle membranes, where it regulates fusion events. Rab5 is localized to early endosomes and regulates the endosomal fusion in endocytosis, whereas Rab7 associates with late endosomes and facilitates the transport from late endosomes to lysosomes (Gutierrez *et al*., 2004; Jager *et al*., 2004) or to the vacuolar membrane (Saito and Ueda, 2009) in animal or plant cells. The GTP-binding and hydrolysis domain, the most conserved domain among Rab GTPases (Nahm *et al*., 2003; Valencia *et al*., 1991), is essential for the regulation of vesicular transport. Mutations in this domain can result in constitutively active or dominant-negative forms of Rab (Valencia *et al*., 1991). Moreover, the post-translational modification signal Cys-X-Cys/CysCys is located at the C-terminal that confers Rab protein prenylation by Rab geranylgeranyl transferase and targeting to specific subcellular membranes (Li *et al*., 2014; Seabra *et al*., 1992).

Some Rab GTPases identified under abiotic stress (Bolte *et al*., 2000; Mazel *et al*., 2004; O'Mahony and Oliver, 1999), such as AtRABG3e (Rab7 homologue), can assist plants to tolerate salt and osmotic stress when overexpressed (Mazel *et al*., 2004). Pathogens can exploit the endocytosis or autophagy systems by modifying Rabs to achieve infection. For instance, *Legionella* modulates Rab1 to induce relocalization and activation (Barr, 2013; Mukherjee *et al*., 2011; Muller *et al*., 2010). Animal viruses are internalized by host cells through the endocytosis pathway in order to target their replication compartments, and Rabs are involved in these intracellular trafficking events. Rab7 is involved in the mediation of the transport of *Semliki forest virus* (SFV) from early endosomes to late endosomes in *Vero* cells. The expression of a dominant-negative Rab7 mutant can cause the accumulation of SFV in early endosomes (Vonderheit and Helenius, 2005). Several forms of Rab, such as Rab5 and Rab7, associate with the NS4B-bound replication complexes of *Hepatitis C virus* (HCV). Knocking down the expression of Rab5 and Rab7 significantly reduces the

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replication of HCV (Manna *et al*., 2010). Compared with research on the involvement of Rabs in animal viral pathogens, reports on the Rabs involved in plant viral pathogens are scarce. The movement protein (MP) of the *Potato mop-top virus*, TGB2, can co-localize with Ara7 (a Rab5 orthologue), suggesting that this MP plays a trafficking role in using the endosomal system (Haupt *et al*., 2005). During *Turnip mosaic virus* (TuMV) infection, the secretory pathway is crucial for the intercellular movement of TuMV; Rab E1d, which is involved in the post-Golgi transport of cargo proteins to the plasma membrane, is essential for this movement (Agbeci *et al*., 2013).

Bamboo mosaic virus (BaMV) belongs to the genus *Potexvirus* and has one single-strand, positive-sense RNA genome. In Taiwan, BaMV infection is prevalent in more than 90% of bamboo plantations, resulting in significant economic loss in the bamboo industry (Lin *et al*., 1992). The BaMV genome contains five open reading frames (ORFs) (Lin *et al*., 1994). ORF 1 encodes a 155-kDa polypeptide, comprising a capping enzyme domain (Huang *et al*., 2004; Li *et al*., 2001a), a 5' triphosphatase and helicase-like domain (Li *et al*., 2001b), and an RNA-dependent RNA polymerase (RdRp) domain (Li *et al*., 1998). ORFs 2–4 encode the MPs of 28, 13 and 6 kDa, respectively. These three ORFs overlap in the genome and are thus referred to as a triple gene block (TGB). ORF 5 encodes the 25-kDa coat protein (CP), which plays a multifunctional role in the viral infection cycle (Hung *et al*., 2014; Lan *et al*., 2010). The three TGB proteins and CP are required for cell-to-cell and long-distance movement (Lin *et al*., 2004, 2006; Tseng *et al*., 2009). Abundant host proteins have been identified to regulate plant virus replication (Gancarz *et al*., 2011; Nagy *et al*., 2012; Sasvari *et al*., 2014). With regard to BaMV, host protein glyceraldehye 3-phosphate dehydrogenase (GAPDH) associates with the viral replication complex and binds specifically to the 3' untranslated region (UTR) of both BaMV viral RNAs and its satellite RNAs. A reduction in expression of GAPDH results in an increase in BaMV accumulation (Prasanth *et al*., 2011). Heat shock protein 90 associates with the viral replication complex and interacts with viral RdRp to regulate the initiation of viral RNA replication (Huang *et al*., 2012b). A putative methyltransferase interacting with BaMV RdRp negatively regulates the accumulation of BaMV (Cheng *et al*., 2009), and a putative Rab GTPase activation protein is involved in the cell-to-cell movement of BaMV (Huang *et al*., 2013).

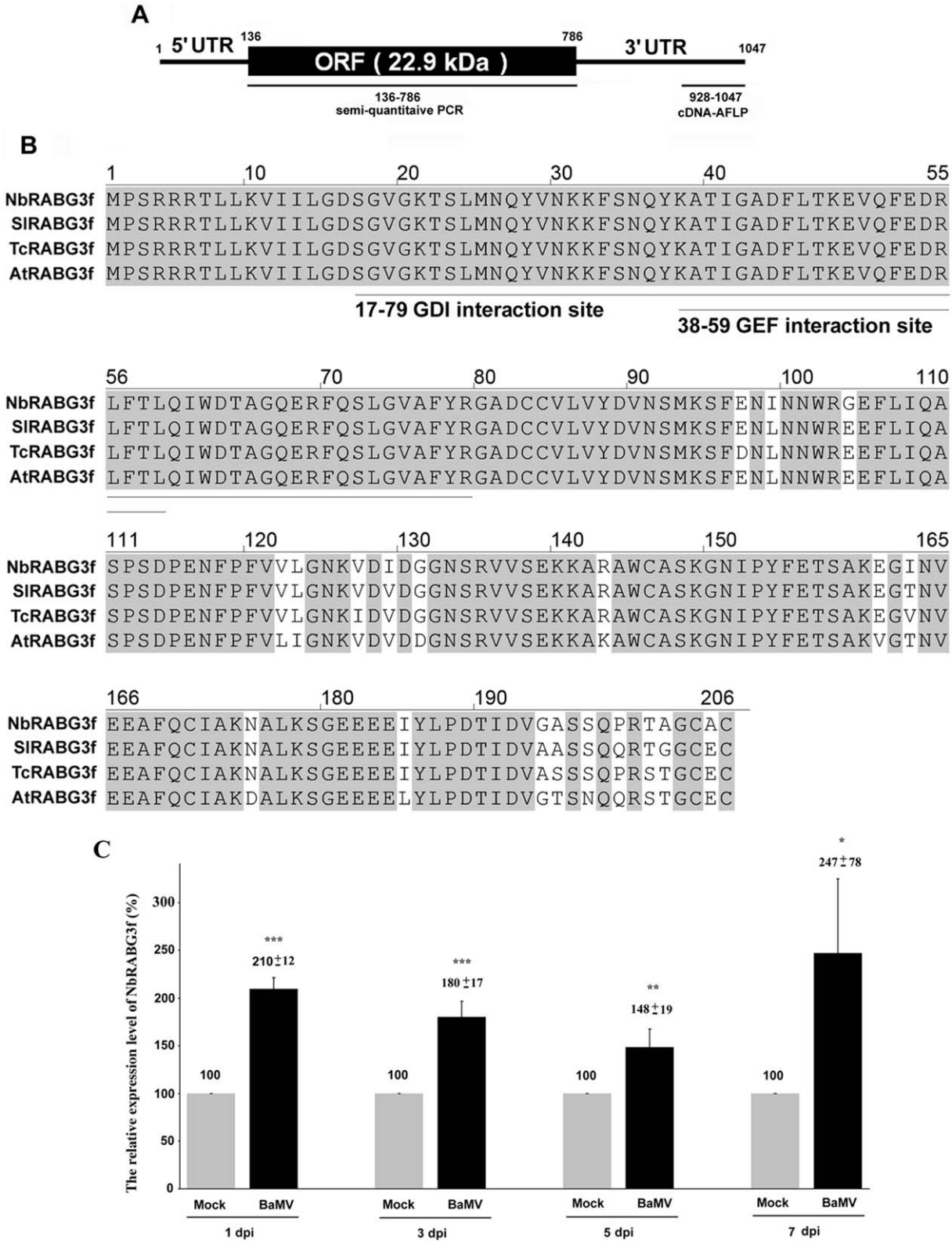
In this study, we focused on an up-regulated cDNA fragment, *ACCT10*, which was identified using a cDNA-amplified fragment length polymorphism (cDNA-AFLP) screening technique (Cheng *et al*., 2010). The sequence of ACCT10 was matched to that of the Rab GTPase and designated as *NbRABG3f*, and the role of this Rab GTPase in the infection of BaMV in *Nicotiana benthamiana* was characterized.

RESULTS

An up-regulated gene induced by BaMV infection encodes a Rab GTPase-like protein

In a previous study, we identified an up-regulated cDNA fragment ACCT10 from BaMV-infected *N. benthamiana* plants (Cheng *et al*., 2010). To obtain the full-length ACCT10 cDNA, we performed 5' and 3' rapid amplification of cDNA ends (RACE) experiments. The cDNA [excluding the poly(A) tail] is 1047 nucleotides in length, comprising 135 and 294 nucleotides in the 5' and 3' UTR, respectively, and an ORF encoding a 206-amino-acid polypeptide that was predicted to be a putative Rab GTPase (Fig. 1A). The *in silico* translated polypeptide of this Rab GTPase-like gene exhibited 96%, 95% and 92% identity with RABG3f-like isoform 1 of *Solanum lycopersicum* (accession number: XP_004230949; SlRABG3f), RABG3f of *Theobroma cacao* (accession number: EOX98047; TcRABG3f) and RABG3f of *Arabidopsis thaliana* (accession number: NP_188512; AtRABG3f), respectively (Fig. 1B). A previous study has predicted that the polypeptide contains various conserved functional domains, including a GTP/Mg^{2+} binding site, GEF interaction site and guanine nucleotide dissociation inhibitor (GDI) interaction site, found in other RABG3f homologues of Rab7 proteins (Marchler-Bauer *et al*., 2011). Thus, we designated this gene as *NbRABG3f*.

Fig. 1 Schematic representations of the full-length cDNA of the *NbRABG3f* gene and the characteristics of this gene. (A) The cDNA of the *NbRABG3f* gene excluding the poly(A) tail is 1047 nucleotides in length comprising the 135 and 294 nucleotides of the 5' and 3' untranslated regions (UTRs), respectively, and 618 nucleotides of the coding region. The coding region is used for the quantification of *NbRABG3f* expression by semi-quantitative polymerase chain reaction (PCR). The cDNA-AFLP fragment is used to knock down the gene expression by virus-induced gene silencing. AFLP, amplified fragment length polymorphism; ORF, open reading frame. (B) The sequence alignment of NbRABG3f with those of SlRABG3f (accession number: XP_004230949 from *Solanum lycopersicum*), TcRABG3f (accession number: EOX98047 from *Theobroma cacao*) and AtRABG3f (accession number: NP_188512 from *Arabidopsis thaliana*). The guanine nucleotide dissociation inhibitor (GDI) and guanine nucleotide exchange factor (GEF) interaction sites are underlined. The conserved sequences of all four species are shaded. The protein sequence was aligned using Vector NTI advanced 11.0 (Explorer). (C) The relative expression levels of *NbRABG3f* were determined by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) using actin as internal control. The total RNAs were isolated from the fourth and fifth leaves of mock- or *Bamboo mosaic virus* (BaMV)-inoculated *N. benthamiana* plants at 1, 3, 5 and 7 days post-inoculation (dpi). The RNAs were reverse transcribed and semi-quantitative PCR amplified with the specific primers according to the sequence of *NbRABG3f*. The numbers above the statistic bars are the means and standard errors compiled from three independent experiments. The data derived from the control (Mock) are set as 1.0 at each time point. The statistical analysis used Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).


A
1 5'UTR 136
ORF (22.9 kDa)
786
3'UTR 1047
136-786
semi-quantitaive PCR
928-1047
cDNA-AFLP
B
1 10 20 30 40 55
NbRABG3f MPSRRRTLLKVIILGDSGVGKTSLMNQYVNKKFSNQYKATIGADFLTKEVQFEDR
SlRABG3f MPSRRRTLLKVIILGDSGVGKTSLMNQYVNKKFSNQYKATIGADFLTKEVQFEDR
TcRABG3f MPSRRRTLLKVIILGDSGVGKTSLMNQYVNKKFSNQYKATIGADFLTKEVQFEDR
AtRABG3f MPSRRRTLLKVIILGDSGVGKTSLMNQYVNKKFSNQYKATIGADFLTKEVQFEDR
17-79 GDI interaction site
38-59 GEF interaction site
56 70 80 90 100 110
NbRABG3f LFTLQIWDTAGQERFQSLGVAFYRGADCCVLVYDVNSMKSFENINNWRGEFLIQA
SlRABG3f LFTLQIWDTAGQERFQSLGVAFYRGADCCVLVYDVNSMKSFENLNNWREEFLIQA
TcRABG3f LFTLQIWDTAGQERFQSLGVAFYRGADCCVLVYDVNSMKSFDNLNNWREEFLIQA
AtRABG3f LFTLQIWDTAGQERFQSLGVAFYRGADCCVLVYDVNSMKSFENLNNWREEFLIQA
111 120 130 140 150 165
NbRABG3f SPSDPENFPFVVLGNKVDIDGGNSRVVSEKKARAWCASKGNIPYFETSAKEGINV
SlRABG3f SPSDPENFPFVVLGNKVDVDGGNSRVVSEKKARAWCASKGNIPYFETSAKEGTNV
TcRABG3f SPSDPENFPFVVLGNKIDVDGGNSRVVSEKKARAWCASKGNIPYFETSAKEGVNV
AtRABG3f SPSDPENFPFVLIGNKVDVDDGNSRVVSEKKAKAWCASKGNIPYFETSAKVGTNV
166 180 190 206
NbRABG3f EEAFQCIAKNALKSGEEEEIYLPDTIDVGASSQPRTAGCAC
SlRABG3f EEAFQCIAKNALKSGEEEEIYLPDTIDVAASSQQRTGGCEC
TcRABG3f EEAFQCIAKNALKSGEEEEIYLPDTIDVASSSQPRSTGCEC
AtRABG3f EEAFQCIAKDALKSGEEEELYLPDTIDVGTSNQQRSTGCEC
C
The relative expression level of NbRABG3f (%)
300
250
200
150
100
50
0
***
210±12
***
180±17
**
148±19
*
247±78
100
100
100
100
Mock
BaMV
Mock
BaMV
Mock
BaMV
Mock
BaMV
1 dpi
3 dpi
5 dpi
7 dpi

A previous study has demonstrated that the mRNA level of *NbRABG3f* is up-regulated following BaMV inoculation (Cheng *et al*., 2010). To confirm this mRNA profile, we extracted the RNAs from mock- and BaMV-inoculated *N. benthamiana* plants from 1 to 7 days post-inoculation (dpi) and quantified *NbRABG3f* mRNA by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). The results revealed that the mRNA level of *NbRABG3f* at 1, 3, 5 and 7 dpi in BaMV-inoculated plants was approximately two-fold higher than that in mock-inoculated plants (Fig. 1C).

Down-regulation of the expression of *NbRABG3f* results in a reduction in the accumulation of BaMV in *N. benthamiana*

The induction of a specific host gene after virus infection may result from the activation of host defence or, by contrast, a host factor required for virus infection. To test these two possibilities, we used *Tobacco rattle virus*-based virus-induced gene silencing (TRV-based VIGS) to knock down the expression of *NbRABG3f* in *N. benthamiana*, followed by BaMV inoculation. The expression level of *NbRABG3f* was reduced to approximately 18% of that in control plants after VIGS (Fig. 2A). We observed no obvious phenotypic change between the control (green fluorescent protein, GFP)- and *NbRABG3f*-knockdown *N. benthamiana* plants (Fig. 2C). The accumulation of BaMV RNAs in *NbRABG3f*-knockdown plants was reduced to approximately 20% of that in control plants; this was determined using real-time RT-PCR at 5 dpi (Fig. 2B). These results indicate that *NbRABG3f* plays a positive role in promoting BaMV accumulation.

Because the accumulation of BaMV in inoculated leaves may have resulted from the effects of viral accumulation within cells and cell-to-cell movement, we used *NbRABG3f*-knockdown protoplasts for BaMV inoculation to exclude the involvement of cell-to-cell movement. The protoplasts were isolated from *NbRABG3f*-knockdown plants and followed by BaMV RNA inoculation. Western blotting showed that BaMV CP accumulation was reduced to approximately 55% of that in *Luciferase* (*Luc*)-knockdown protoplasts (Fig. 3A). The accumulation of both positive and negative strands of BaMV genomic RNA was also measured by Northern blotting (Fig. 3B and 3C, respectively), which revealed that this accumulation was approximately 60% of that in the control (Fig. 3D). These results indicate that reducing the expression of *NbRABG3f* reduces the accumulation of BaMV in the protoplasts. Therefore, the polypeptide encoded by *NbRABG3f* could positively regulate the accumulation of BaMV within cells.

Because knocking down the expression of *NbRABG3f* in leaves had a greater effect (20% of that in the control) than that in protoplasts (60% of that in the control), we further inoculated GFP-carrying BaMV (Lin *et al*., 2004) onto *N. benthamiana* leaves and traced the viral movement with a fluorescent microscope. The GFP foci production by BaMV infection from *NbRABG3f* knocked-down leaves was slightly, but significantly, smaller than that of control leaves (Fig. 4). However, the reduction in viral replication observed in protoplasts (Fig. 3) may compound the reduction effect obtained in leaves.

Expression of NbRABG3f in *N. benthamiana* enhances the accumulation of BaMV

To substantiate the positive regulation of *NbRABG3f* in the accumulation of BaMV in *N. benthamiana*, we further transiently expressed the GFP-fused NbRABG3f (GFP-NbRABG3f) into *N. benthamiana* (Fig. 5A), followed by BaMV inoculation. Compared with the GFP expression control, the expression of GFP-NbRABG3f enhanced the accumulation of BaMV to 146% (Fig. 5B), providing direct evidence that the polypeptide NbRABG3f facilitates the accumulation of BaMV.

To further investigate whether the GTPase activity of NbRABG3f is involved in BaMV infection, we transiently expressed the mutant GFP-NbRABG3f/T22N, where a threonine at position 22 was changed to asparagine, which fixes NbRABG3f to a GDP-bound form (Nahm *et al*., 2003). The expression of GFP-NbRABG3/T22N did not markedly enhance the accumulation of BaMV (Fig. 5B). The results indicate that the GTPase activity of NbRABG3f is involved in facilitating the accumulation of BaMV.

The membrane-targeting domain on the C-terminus of Rabs is necessary for Rab GTPases to associate with vesicles. To inspect whether the membrane-targeting ability of NbRABG3f is crucial for BaMV accumulation, we constructed two deletion mutants, *GFP-NbRABG3f/ΔC3* and *GFP-NbRABG3f/ΔC10*, to remove the C-terminal three and 10 amino acids, respectively. The protein extracts from plants transiently expressing the wild-type and deletion mutants of GFP-NbRABG3f were fractionated and examined using Western blotting to ensure that the deletion constructs were no longer associated with the membrane after expression in plants. The results indicated that GFP-NbRABG3f was mainly membrane associated; by contrast, the GFP-NbRABG3f/ΔC3 and GFP-NbRABG3f/ΔC10 mutants were mostly solubilized (Fig. 5C). Because the C-terminus of Rabs contains a potential prenylation site, GFP-NbRABG3f/ΔC3 and GFP-NbRABG3f/ΔC10, with the deletion of the prenylation site at the C-terminus, were revealed in the soluble fraction. The accumulation of BaMV CP in *GFP-NbRABG3f/ΔC3*-infiltrated leaves was markedly reduced to approximately 75% of that in the GFP-infiltrated control (Fig. 5B). These results indicate that the membrane-targeting ability is crucial for NbRABG3f to facilitate the accumulation of BaMV.

NbRABG3f induces a vesicle-like structure from the Golgi membrane

The results of biochemical analysis revealed that NbRABG3f is a membrane-associated protein, whereas NbRABG3f/ΔC3 is a soluble protein from which the membrane-targeting signal has

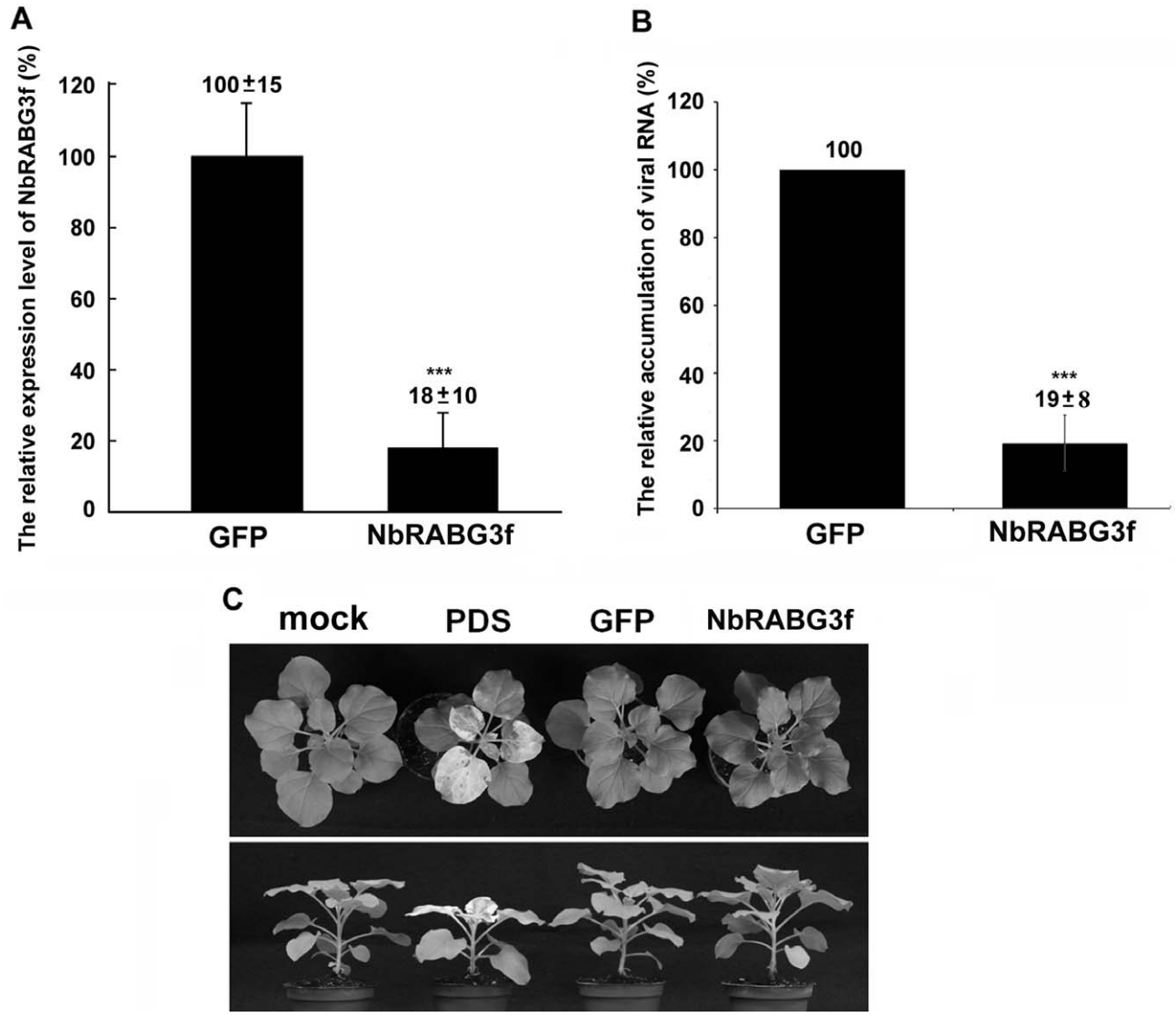


Fig. 2 The accumulation levels of *Bamboo mosaic virus* (BaMV) in *NbRABG3f*-knockdown plants. (A) The accumulation of *NbRABG3f* mRNA in *GFP*- or *NbRABG3f*-knockdown *Nicotiana benthamiana* plants at 14 days post-silencing determined by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). (B) The relative accumulation levels of BaMV viral RNA in *GFP*- or *NbRABG3f*-knockdown *N. benthamiana* plants, followed by BaMV inoculation on the fourth and fifth leaves above the infiltrated leaves and harvested at 5 days post-inoculation (dpi) and determined by real-time RT-PCR. The numbers above each statistic bar are the means and standard errors compiled from three independent experiments. Actin was used as internal control for normalization. The data derived from the GFP-knockdown control plants were set as 100% for comparison. (C) The phenotypes of the knockdown plants. The 1-month-old *N. benthamiana* plants were subjected to the knockdown experiments using the *Tobacco rattle virus* (TRV)-based silencing system. The green fluorescent protein (GFP) gene was used as negative control and phytoene desaturase (PDS) was used as a control to trace the knockdown procedure.

been removed (Fig. 5C). To confirm these results *in vivo*, we used confocal microscopy to visualize the distribution of these two proteins in *N. benthamiana* leaves (Fig. 6A) and protoplasts (Fig. 6B). The expressed GFP-NbRABG3f protein aggregated around vesicle-like structures (Fig. 6); by contrast, NbRABG3f/ΔC3 lost its membrane-targeting signal, and was distributed in the cells in a manner that was similar to that of GFP expression in cells (Fig. 6). According to the functional properties of Rabs in the vesicle trafficking model (van der Bliek, 2005), Rab-GDPs associate with the membrane of the donor compartment and interact with GEF to form Rab-GTP. Subsequently, Rab-GTP can trigger vesicle formation and trafficking to the membrane of the acceptor compartment. Therefore, the GDP-bound form of Rab can be used to trace the donor compartment (Sieczkarski and Whittaker, 2002). The images of the fixed Rab-GDP mutant revealed that NbRABG3f/T22N co-localized with the Golgi marker (Fig. 6). These results indicate that the NbRABG3f-associated vesicles could be initiated from the Golgi membrane.

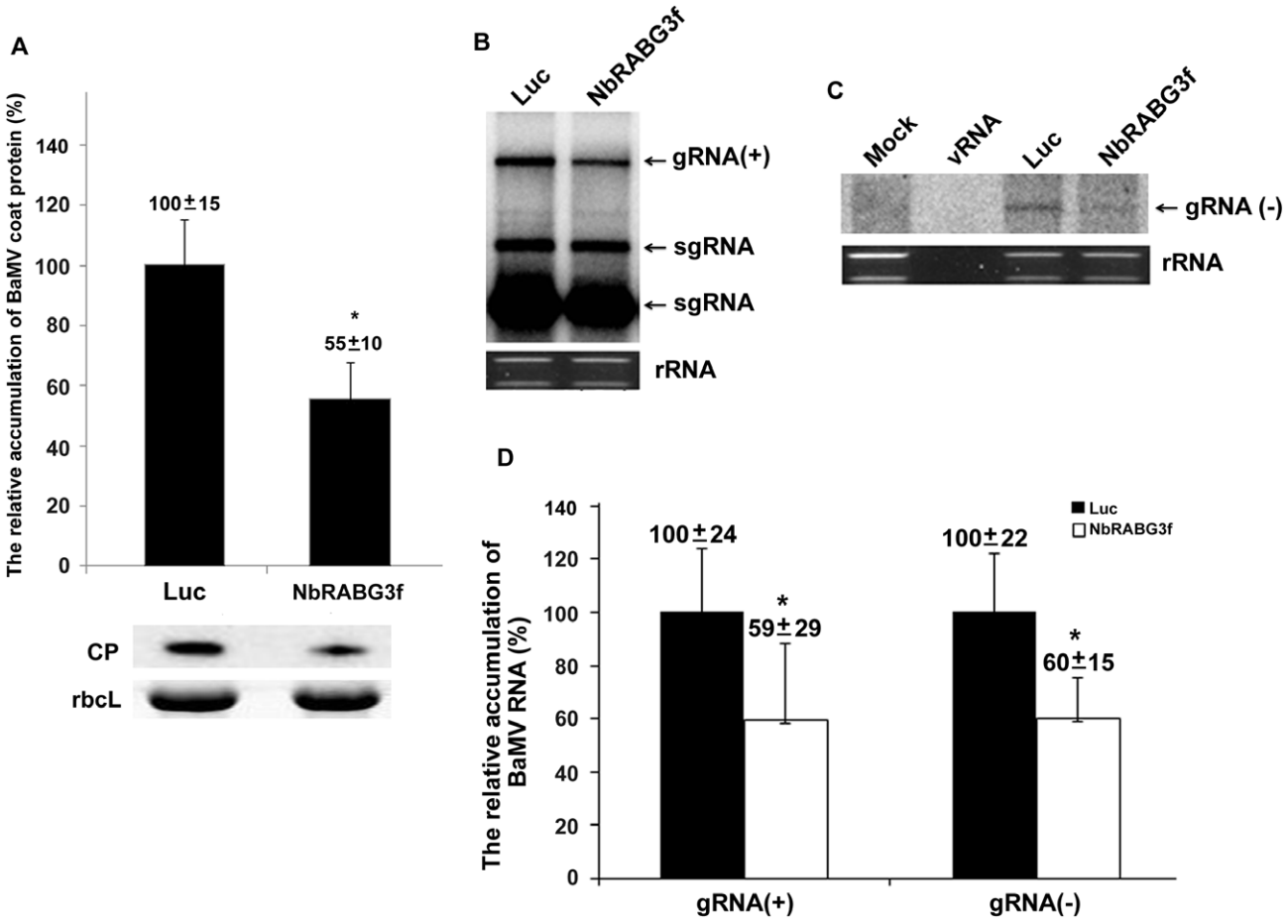


Fig. 3 The accumulation levels of *Bamboo mosaic virus* (BaMV) in the *NbRABG3f*-knockdown protoplasts. The knockdown protoplasts were isolated from *Luciferase* (Luc)- or *NbRABG3f* (NbRABG3f)-knockdown *Nicotiana benthamiana* plants. (A) The BaMV coat protein (CP) accumulation level was determined by Western blotting. rbcL was used as internal control. Northern blotting of BaMV plus-strand (B) and minus-strand (C) viral RNA accumulation in *Luciferase* (Luc)- or *NbRABG3f* (NbRABG3f)-knockdown *N. benthamiana* protoplasts at 24 h post-BaMV viral RNA inoculation. gRNA, genomic RNA; sgRNA, subgenomic RNA; vRNA, viral RNA. Luc control was set as 100% for comparison. (D) The relative accumulation of BaMV genomic RNA determined by Northern blotting derived from (B) and (C). rRNA was used as the loading control. The numbers above each statistic bar are the means and standard deviations compiled from two independent experiments with triplicates in each experiment.

To analyse whether the subcellular localization of GFP-NbRABG3f was changed after BaMV inoculation, we transiently expressed GFP-NbRABG3f or its derivatives followed by BaMV inoculation. The protoplasts were isolated from the inoculated leaves at 3 dpi and inspected using confocal microscopy (Fig. S1, see Supporting Information). The results indicated no observable difference between BaMV-inoculated (Fig. 6B) and non-inoculated (Fig. S1) protoplasts.

DISCUSSION

In this study, we used a TRV-based VIGS system to knock down the expression of *NbRABG3f* in *N. benthamiana*, reducing the accumulation of BaMV in plants (Fig. 2) and protoplasts (Fig. 3). In addition, transiently expressed NbRABG3f in *N. benthamiana* leaves enhanced the accumulation of BaMV, but not that of the GTPase active site mutant (Fig. 5B). These results indicate that NbRABG3f combined with functional GTPase activity is crucial in BaMV accumulation within cells, because the fixed Rab-GDP lost its activity and exerted no effect on BaMV accumulation (Fig. 5B). Furthermore, functional targeting to the vesicle membrane is crucial in facilitating the accumulation of BaMV. Mutants without a C-terminal prenylation sequence mainly distributed in the cytoplasm rather than the vesicle-like structure (Fig. 6) and did not facilitate BaMV accumulation (Fig. 5B). Overall, these results suggest that NbRABG3f is required for the efficient accumulation of BaMV, and NbRABG3f possibly initiates vesicle formation from the Golgi membrane (Fig. 6).

In addition, we revealed that BaMV viral RNA accumulation was significantly inhibited (20% of that in the control) after knock-

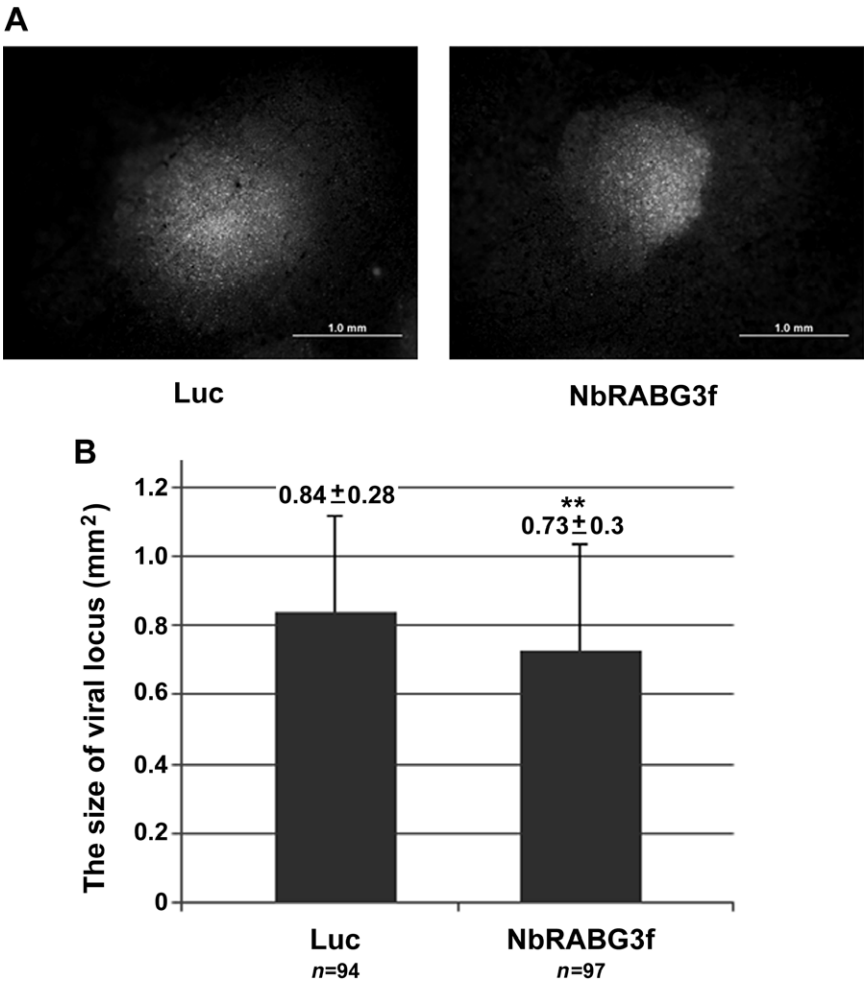


Fig. 4 The effects of NbRABG3f knockdown on *Bamboo mosaic virus* (BaMV) infection. The fourth leaf above the infiltrated leaves was inoculated with 500 ng of BaMV-GFP particles and measured at 5 days post-inoculation (dpi). (A) The areas of green fluorescent protein (GFP) foci in the inoculated leaves of the *Luc*- and *NbRABG3f*-knockdown plants were determined under the fluorescent microscope after inoculation with the virion derived from pCBG carrying GFP. Bar, 1.0 mm. (B) Statistical analysis of the results derived from (A). *x*-axis is the GFP focus size (mm^2). The numbers shown above the statistic bar are the average and standard deviations of 94 and 97 foci from *Luc*- and *NbRABG3f*-knockdown plants, respectively.

ing down the expression of *NbRABG3f* in *N. benthamiana* leaves, but the effect was less profound in protoplasts (60% relative to that in the control). Although less viral RNA replication in cells could result in less cell-to-cell movement and further accumulation in neighbouring cells, the differential expression levels of viral RNA in leaves and protoplasts indicated that the effect of NbRABG3f on intracellular and intercellular trafficking of BaMV may play a role in the accumulation of BaMV. According to the observation that BaMV CP and genomic RNA accumulated to similar levels after knocking down the expression of NbRABG3f, the function of NbRABG3f may be involved in BaMV genomic RNA replication rather than CP translation (Fig. 3).

Furthermore, the results of the NbRABG3f-knockdown experiment (Fig. 3) suggested that NbRABG3f mainly affected minus-strand RNA synthesis and created a fixed ratio of plus-strand RNA reduction (Chen *et al*., 2005; Wang and Nagy, 2008). The detailed mechanism of this effect must still be demonstrated in an *in vitro* experiment.

Previous studies have shown that several Rab GTPases are involved in animal or human virus replication (Manna *et al*., 2010), intracellular transportation (Vonderheit and Helenius, 2005) and intercellular spread (Agbeci *et al*., 2013). In plant viruses, a Rab protein of *Arabidopsis* is involved in the intercellular movement of TuMV (Agbeci *et al*., 2013), a Rab activator protein (NbRabGAP 1) regulates the movement of BaMV, but not its replication (Huang *et al*., 2013), and a Rab GDI of *Arabidopsis* associates with the *Tobacco mosaic virus* 126-kDa replication protein and relocalizes the interacted GDI from the cytoplasm to the endoplasmic reticulum (Kramer *et al*., 2011). These findings indicate that viruses may use host proteins by changing their function or location for their own purpose (Huang *et al*., 2012a). However, we did not observe a significant subcellular localization change of NbRABG3f between BaMV non-inoculated and inoculated protoplasts (Figs 6B and S1, respectively).

Most animal viruses enter their host cells through the endocytosis pathway (Mudhakir and Harashima, 2009). Although plant virus entry into host cells may occur through distinct mechanisms, the intracellular movement of plant viruses to the specific subcellular locations could be similar (Patarroyo *et al*., 2013). *Arabidopsis* RABG3 (Rab7 analogue) is involved in the vacuolar transport from the pre-vacuolar compartment to the vacuole (Woollard and Moore, 2008). The MP of *Cauliflower mosaic virus*

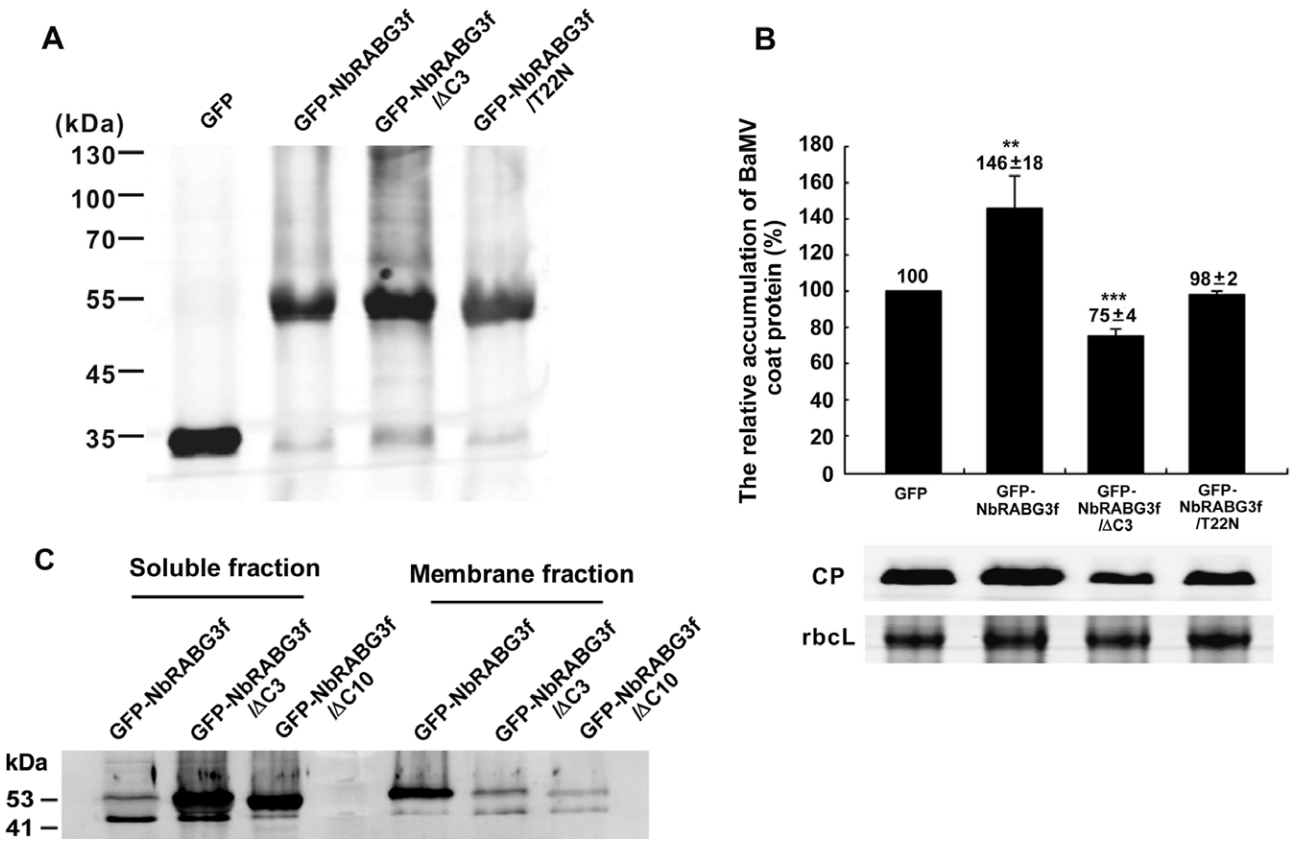


Fig. 5 The accumulation level of *Bamboo mosaic virus* (BaMV) in *Nicotiana benthamiana* plants expressing *NbRABG3f* or its derivatives. The third, fourth and fifth leaves at the six-leaf stage were *Agrobacterium* infiltrated and followed by BaMV inoculation at 1 day post-infiltration, and the inoculated leaves were harvested at 2 days after virus inoculation. (A) Western blotting detection of GFP-, GFP-NbRABG3f-, GFP-NbRABG3f/ΔC3- or GFP-NbRABG3f/T22N-expressing *N. benthamiana* plants with anti-GFP antibody. (B) The relative accumulation levels of the BaMV coat protein (CP) in the transiently expressed plants as indicated. The numbers above each statistic bar are the means and standard errors compiled from three independent experiments. The data derived from the green fluorescent protein (GFP) control plants are set as 100% for comparison. (C) Western blotting analysis of the fractionation of the extracts isolated from the transiently expressed plants as indicated using the anti-GFP antibody.

(CaMV) co-localizes with AtRAB-F2b, a GTPase that delivers proteins from late endosomes to vacuoles for degradation. Thus, AtRAB-F2b is believed to deliver excess CaMV MP to the vacuole for degradation (Carluccio *et al*., 2014). However, in the case of BaMV in *N. benthamiana*, because knocking down the expression of NbRABG3f reduced BaMV accumulation within cells, BaMV might use NbRABG3f to deliver its own cargo to its replication destination rather than to the vacuole for degradation. Nevertheless, co-localization of the BaMV replication complex and different organelle markers is needed to dissect this pathway in more detail.

In this study, we observed that NbRABG3f aggregated around the vesicle-like structure and the GDP-fixed form T22N was trapped in the Golgi (Figs 6 and S1), unlike RABG3f studied in *A. thaliana* (Cui *et al*., 2014). Recently, Li *et al*. (2014) have demonstrated that hypervariable C-terminal domains of Rab7 proteins are involved in correct membrane targeting and interaction with Rab-interacting lysosomal proteins. Protein sequence comparisons between two RABG3fs revealed only a 50% identity for the last 12 amino acids of the C-terminus, compared with a 92% identity for the full-length protein (data not shown). Therefore, NbRABG3f identified in this study might have a subcellular localization or function different from that of AtRABG3f.

Unlike the wild-type protein, NbRabG3f/T22N overexpression did not promote the accumulation of BaMV (Fig. 5), indicating that the functional GTPase activity of NbRABG3f must be crucial in facilitating the accumulation of BaMV, possibly by delivering BaMV or the required elements to the replication sites. The switch between the GTP- and GDP-bound forms of Rabs is critical for vesicle trafficking. The GDP-bound NbRABG3f/T22N trapped at the Golgi membrane thus could not deliver the cargo required for BaMV replication to an appropriate subcellular location, and therefore did not support the BaMV replication that was obtained with NbRABG3f. However, a similar mutation in other Rabs has various effects on the foot-and-mouth disease virus; specifically, Rab5 and Rab11, with fixed GDP-bound mutations, reduce virus infection by 80% and 35%, respectively, whereas the same

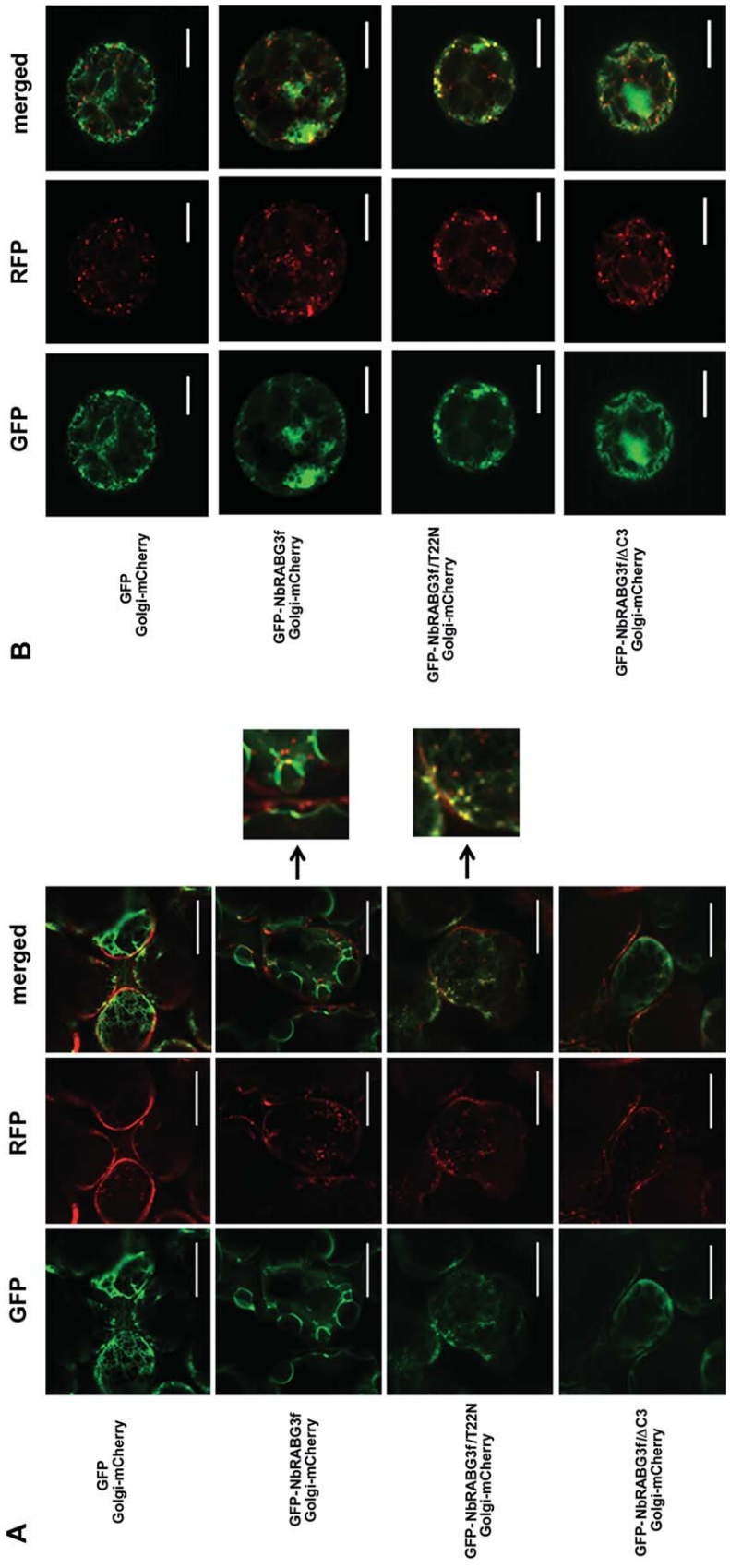


Fig. 6 The subcellular localization of GFP-NbRABG3f and its derivatives in *Nicotiana benthamiana*. The green fluorescent protein (GFP)-fused NbRABG3f and its derivatives were transiently expressed in *N. benthamiana* leaves (A) or protoplasts (B), and examined by confocal microscopy. mCherry fused with a Golgi signal peptide is transiently expressed in leaves and is used as the Golgi marker. The signals of GFP, GFP-NbRABG3f, GFP-NbRABG3f/ΔC3 and GFP-NbRABG3f/T22N are shown in green. mCherry-Golgi is shown in red and the co-localized signal is shown in yellow. The scale bar is 10 μm. RFP, red fluorescent protein.

Table 1 Primers used in this study.

Name of the primer	Sequence of the primer
ACCT10 5' RACE-1	GAACTCTGTTGGTGCTATTTATGTTATTA
ACCT10 3' RACE-2	GAACCTTAAGATTTGAAGG
BaMV(–) 2002	ATGTATCACGGAAATAAGAGTT
BaMV(+) 1766	CACATCCGGCACTTACCA
Actin/F	GATGAAGATACTCACAGAAAGA
Actin/R	GTGGTTTCATGAATGCCAGCA
ACCT10 3'RACE	TAATAAATAAATAGCACCAACAGAGTTC
ACCT10/R	TAACTGAATATGGTTTCTACAATT
*Sac*I/NbRab/F	ACGAGCTCATGCCTTCACGCCGGCGAA
*Sac*I/NbRab/R	ACGAGCTCTTAACACGCGCATCCAGCTGT
NbRabT22N/R-1	CATCAACGAGTTCTTCCCAAC
*Sac*I/Rab del3/R	ACGAGCTCTTATCCAGCTGTCCTCGGCTG
*Sac*I/Rab del10/R	ACGAGCTCTTAGCTAGCGCCAACATCAATG

mutations of Rab4 and Rab7 do not reduce virus infection (Johns *et al*., 2009). Through these analyses, the intracellular transport of viruses can be resolved. Therefore, further research dissection of other NbRABs is necessary to understand the pathway through which BaMV is transported intracellularly.

EXPERIMENTAL PROCEDURES

Plants and viruses

Nicotiana benthamiana was grown in a growth chamber at 28 °C with 16 h light/8 h dark, as described elsewhere (Cheng *et al*., 2010). BaMV severe strain (BaMV-S) (Lin *et al*., 1994) was used for inoculation.

Total RNA and mRNA extraction

After virus inoculation or VIGS treatment, total RNAs were extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, from 100 mg of *N. benthamiana* leaves. The mRNA was purified from the isolated total RNAs using Dynabeads Oligo $(dT)_{25}$ (Dynal A.S., Oslo, Norway), as described elsewhere (Cheng *et al*., 2010), to clone the full-length cDNA of *NbRABG3f*.

RT-PCR

For the detection of viral RNA accumulation, BaMV-specific primer BaMV(–) 2002 was used to generate cDNA with the SuperScript III First-Strand Synthesis System (Invitrogen) and followed by the addition of primer BaMV(+) 1766 for semi-quantitative PCR or real-time PCR. The internal control actin was amplified with actin-specific primers Actin/F and Actin/R (Table 1). All of the PCRs were set with the conditions 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. For semi-quantitative RT-PCR, the PCR products were separated on a 1% agarose gel, stained with ethidium bromide and then quantified by ImageJ. Real-time PCR was performed using GoTaq® qPCR Master Mix (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Northern blotting

The total RNAs extracted from BaMV-inoculated leaves or protoplasts were glyoxylated and separated on a 1% agarose gel, and transferred to the nitrocellulose membrane, as described previously (Lin *et al*., 1992). The plus and minus RNA probes of BaMV were prepared from the plasmid pBL2.6 (Tsai *et al*., 1999), digested with *Hind*III to generate a 600-nucleotide transcript complementary to the 3'-terminal region of the BaMV genome, and plasmid pBaMV.S (Lin *et al*., 2005), digested with *Kpn*I to generate a 500-nucleotide transcript complementary to the 3'-terminal region of the BaMV minus genome, respectively.

Full-length cDNA cloning of *NbRABG3f*

To clone the full-length *NbRABG3f* cDNA, a SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) was used to extend the sequences for both 5' and 3' ends. Briefly, for 5' RACE, the gene-specific primer (ACCT10 5' RACE-1, Table 1) was designed for RT, followed by PCR with the GeneRacer™ 5' Nested Primer and ACCT10 5' RACE-1. For 3' RACE, the GeneRacer™ 3' Nested Primer was used for RT, followed by PCR with the gene-specific primer ACCT10 3' RACE-2 and GeneRacer™ 3' Nested Primer provided by the kit.

Constructs

The TRV-based knockdown system was kindly provided by Dr David Baulcombe (Department of Plant Sciences, University of Cambridge, UK). The primers ACCT10 3' RACE and ACCT10/R were used to amplify the fragment identified from cDNA-AFLP by PCR and cloned into pGEM-T Easy vector (Promega). The fragment was derived from the plasmid digested with *Eco*RI and subcloned into pTRV2 vector. The resulting plasmid was transformed into *Agrobacterium tumefaciens* C58C1 strain for the knock-down experiment in *N. benthamiana*, as described previously (Cheng *et al*., 2010). For the transient expression, the cDNA fragment containing the ORF of *NbRABG3f* was amplified with primers *Sac*I/NbRab/F and *Sac*I/NbRab/R. To clone the dominant-negative mutant of the *NbRABG3f* mutant, *NbRABG3f*/*T22N*, the primers NbRabT22N/R-1 and SacI/NbRab/F were used to make the mega-primer. The mega-primer was gel purified and used with the reverse primer *Sac*I/NbRab/R for PCR to produce the full-length mutant. *NbRABG3f* and *NbRABG3f*/*T22N* were cloned into pBI-mGFP (Cheng *et al*., 2013a) with the *Sac*I site in which GFP was fused to the N-terminus of the desired proteins (designated as GFP-NbRABG3f and GFP-NbRABG3f/T22N, respectively). The C-terminal deletion mutants *NbRABG3f*/*ΔC3* and *NbRABG3f*/*ΔC10* were cloned by PCR amplification with the primers *Sac*I/Rab/F and *Sac*I/Rab del3/R or del10/R, respectively, followed by the same procedure as described above.

Agrobacterium transformation

Agrobacterium strain C58C1 competent cells were used for transformation modified from the freeze–thaw method (Hofgen and Willmitzer, 1988). Briefly, 1 μg of DNA was added to 100 μL *Agrobacterium* competent cells, the mixture was frozen in liquid nitrogen for 5 min and incubated at 37 °C for another 5 min, followed by the addition of 1 mL of 2xYT medium and

incubation at 28 °C with gentle shaking for 2–4 h. The transformed cells were then plated onto a kanamycin-containing plate and incubated at 28 °C for 2–3 days.

VIGS and virus inoculation

For VIGS, the TRV-based knockdown system was used, as described previously (Cheng *et al*., 2013b). Briefly, agrobacteria containing plasmid TRV1 and TRV2 derivatives were mixed in a 1 : 1 ratio and infiltrated into the second, third and fourth true leaves of five-leaf stage *N. benthamiana*. The infiltrated plants were grown at 28 °C for 10 days to knock down the expression of the target gene. Phytoene desaturase (PDS) was used as a marker gene that displayed a photobleaching character after knocking down the expression to monitor the VIGS process (Liu *et al*., 2002). The fourth and fifth (eighth and ninth true leaves of the plant) leaves above the infiltrated leaves were inoculated with 500 ng of BaMV virion and harvested at 5 dpi. The inoculated leaves of each plant and at least three plants were used for each treatment and mixed for further analysis. All the experiments were repeated independently at least three times.

Transient expression of *NbRABG3f* and its derivatives

The procedure of transient expression has been described previously (Cheng *et al*., 2013b). In general, after induction with acetosyringone, agrobacteria containing *NbRABG3f* or its derivatives were mixed with *Agrobacterium* harbouring the silencing suppressor HcPro (Chiu *et al*., 2010) in a 1 : 1 ratio and infiltrated onto the third, fourth and fifth leaves of *N. benthamiana* at the six-leaf stage. One day after agro-infiltration, 150 ng of BaMV virion was inoculated onto the infiltrated leaves and harvested at 2 dpi.

Measurement of GFP foci

Approximately 500 ng of BaMV vector carrying the GFP gene (Lee *et al*., 2011) were inoculated onto the fourth leaf above the infiltrated leaves of *Luc*- (control) or *NbRabG3f*-knockdown *N. benthamiana* plants. The green fluorescent foci on the inoculated leaves were captured at 5 dpi with an Olympus (Tokyo, Japan) IX71 inverted microscope (Cheng *et al*., 2013b). The green fluorescent area at the BaMV foci was determined by ImageJ software.

Western blot analysis

Total proteins were extracted from BaMV-inoculated leaves and separated on 12% sodium dodecylsulfate (SDS)-polyacrylamide gels. The viral CPs of BaMV were detected using rabbit anti-BaMV CP polyclonal antibodies, analysed by Western Lightning Plus-ECL (Perkin Elmer, Shelton, CT, USA) and quantified by UVP VisionWorkLS version 5.5.4 software (Upland, CA, USA). The relative level of Rubisco large subunit (rbcL) in gels stained with Coomassie brilliant blue was used for normalization. The expression of GFP-NbRABG3f and its derivatives was detected with anti-GFP antibody.

Protoplast isolation and viral RNA inoculation

For knockdown experiments, protoplasts were isolated from *NbRABG3f*-knockdown *N. benthamiana* 14 days after *Agrobacterium* infiltration. Approximately 5×10^5 cells were inoculated with 1 μg of BaMV viral RNA plus 20% of polyethylene glycol 6000, as described previously (Tsai *et al*., 1999), and cultured at room temperature for 24 h under constant light.

Separation of cytoplasm and membrane fractions

The fractionation procedure was performed as described previously (Cheng *et al*., 2013b), except that 2% Triton X-100 was added for the solubilization of NbRABG3f instead of 2% SDS. Briefly, 0.5 g of transiently expressed *N. benthamiana* leaves were ground with liquid nitrogen, and 1 mL of pre-chilled buffer A (Cheng *et al*., 2013b) was added and centrifuged at 12 000 ***g*** for 10 min at 4 °C. After centrifugation, the supernatant was transferred to a new 1.5-mL microtube. The pellet containing the membrane fraction was washed twice with 2 mL of buffer A in a 12 000 g centrifugation step for 10 min each time at 4 °C. The pellet was then dissolved in 1 mL of buffer A with 2% Triton X-100 and stirred for 30 min at 4 °C. The solubilized membrane proteins were collected by 12 000 ***g*** centrifugation for 10 min at 4 °C.

Localization by laser scanning confocal microscopy

Agrobacteria containing pBIN-GFP (GFP only), pBIN-GFP-NbRABG3f, pBIN-GFP-NbRABG3f/ΔC3, pBIN-GFP-RABG3f/T22N, pCD3-967 (Golgi-mCherry marker) (Nelson *et al*., 2007) and pBIN61-HcPro were cultured and induced with 450 μM acetosyringone in 10 mM $MgCl_2$ to a final optical density at 600 nm (OD_{600}) = 1. *Agrobacterium* containing the GFP construct (pBIN-GFP, pBIN-GFP-NbRABG3f, pBIN-GFP-NbRABG3f/ΔC3 or pBIN-GFP-RABG3f/T22N) was mixed with that containing pBIN61-HcPro and pCD3-967 in a 1 : 1 : 1 ratio and infiltrated into *N. benthamiana* leaves. Images were obtained with an Olympus Fluoview FV1000 laser scanning confocal microscope using 488- and 543-nm laser excitation for GFP and mCherry, respectively, at 3 days post-infiltration.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 The subcellular localization of GFP-NbRABG3f and its derivatives in *Nicotiana benthamiana* protoplasts after *Bamboo mosaic virus* (BaMV) inoculation. BaMV virion was inoculated onto the fourth leaf 6 h after *Agrobacterium* infiltration and harvested at 3 days post-inoculation (dpi) for protoplast isolation and examined by confocal microscopy. mCherry-Golgi is shown in red and the signals of green fluorescent protein (GFP), GFP-NbRABG3f, GFP-NbRABG3f/ΔC3 and GFP-NbRABG3f/T22N are shown in green. The co-localized signal is shown in yellow. The scale bar is 10 μm.