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A putative Rab-GTPase activation protein from *Nicotiana benthamiana* is important for *Bamboo mosaic virus* intercellular movement

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ABSTRACT

The cDNA-amplified fragment length polymorphism technique was applied to isolate the differentially expressed genes during *Bamboo mosaic virus* (BaMV) infection on *Nicotiana benthamiana* plants. One of the upregulated genes was cloned and predicted to contain a TBC domain designated as NbRabGAP1 (Rab GTPase activation protein 1). No significant difference was observed in BaMV accumulation in the *NbRabGAP1*-knockdown and the control protoplasts. However, BaMV accumulation was 50% and 2% in the inoculated and systemic leaves, respectively, of the knockdown plants to those of the control plants. By measuring the spreading area of BaMV infection foci in the inoculated leaves, we found that BaMV moved less efficiently in the *NbRabGAP1*-knockdown plants than in the control plants. Transient expression of the wild type NbRabGAP1 significantly increases BaMV accumulation in *N. benthamiana*. These results suggest that NbRabGAP1 with a functional Rab-GAP activity is involved in virus movement.

Introduction

Plant viruses need to undergo cell-to-cell movement in the form of virion or viral ribonucleoprotein complex (vRNP) via the plasmodesmata (PD) in order to spread within a plant (Hofmann et al., 2007). The viral-encoded proteins responsible for this process include both classically-defined movement proteins (MPs) and additional non-classical ancillary viral proteins such as the replicase of *Tobacco mosaic virus* (TMV) (Hirashima and Watanabe, 2001, 2003) and the VPg of potyvirus (Dunoyer et al., 2004). In addition, involvement of host factors and subcellular structures are required for virus movement (Benitez-Alfonso et al., 2010; Niehl and Heinlein, 2011; Schoelz et al., 2011; Scholthof, 2005; Taliansky et al., 2008). Host proteins of diverse functions have been identified as viral intercellular trafficking factors that interact with the MP and/or the PD (Harries et al., 2010; Niehl and Heinlein, 2011; Yoshii et al., 2008; Zavaliev et al., 2010). The cytoskeleton system, including the associated motors, has been shown to be involved in transporting the viral components to the PD, although conflicting conclusions have been reached for some viruses (Avisar et al., 2008; Liu and Nelson, 2013; Prokhnevsky et al., 2005). Additionally, the endomembrane system has been reported to play roles directly or indirectly in viral trafficking (Pena and Heinlein, 2012; Tilsner et al., 2012). The formation of the MP-containing tubules that relies on the secretory pathway was shown in cells infected with *Grapevine fanleaf virus* (Laporte et al., 2003). The MPs of *Potato mop-top virus* (PMTV), TGBp2 and TGBp3, were shuttled back to the secretory pathway via endocytosis after they increased the size-exclusion limit of PD (Haupt et al., 2005).

The best-known regulators for the formation of endomembrane vesicles are Rabs, a family of small guanosine triphosphatases (GTPases), and their regulator proteins, the Rab-GTPase activating proteins (Rab-GAPs). Rabs, found in all eukaryotes, are known to participate in all aspects of intracellular vesicle trafficking including vesicle budding, targeting, docking, and fusion (Cherfils and Zeghouf, 2013; Johansen et al., 2009; Mizuno-Yamasaki et al., 2012). In the GTP-bound form, Rabs are active and are able to trigger downstream vesicle trafficking pathways through their effectors. Subsequently, Rabs, the GTP-bound active form, are modified to the GDP-bound inactive form, and are then ready for the next cycle. The GTPases need the assistance of Rab-GAPs to become fully inactive (Cherfils and Zeghouf, 2013). Rab-GAPs contain a catalytic domain called the TBC (derived from Tre-2, Bub2, and Cdc16) domain that accelerates the GTP hydrolysis reaction by Rabs/GTP, which turns off the activity of Rab after the completion of vesicle trafficking (Cherfils and Zeghouf, 2013).

Bamboo mosaic virus (BaMV) is a flexuous-rod, positive-sense RNA virus belonging to the *Potexvirus* genus of the *Flexiviridae*

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family. The RNA genome of approximately 6.4 kb contains five open reading frames (ORF1–ORF5). ORF1 encodes a 155-kDa replicase for RNA synthesis (Li et al., 1998). ORF2 to ORF4 organized as a triple gene block (TGB) in the genome, which participate in intra- and intercellular movements of the virus (Lin et al., 2004, 2006). All the three TGB proteins and coat protein are essential for BaMV cell-to-cell movement. TGBp1 was further discovered to have the RNA-binding and NTPase activities (Hsu et al., 2004; Lin et al., 2004). TGBp2 localized at the endoplasmic reticulum (ER) membrane was critical for both cell-to-cell movement and systemic movement (Tseng et al., 2009). TGBp3 was suggested to have a sorting signal for targeting to the ER tubules, which was required for cell-to-cell spread (Wu et al., 2011). ORF5 encodes the coat protein for viral assembly.

In the present study, we have identified a TBC domain containing gene from *N. benthamiana*, suggesting that it functions as a Rab-GAP and designated as *NbRabGAP1*. The functional GAP activity is necessary to support the efficient movement of BaMV in *N. benthamiana* plants.

Results

An upregulated gene after BaMV infection encodes a TBC domain-containing protein

One of the upregulated cDNA fragments (194-nt) identified by the cDNA-AFLP technique (Cheng et al., 2010) was cloned and sequenced. The full-length gene was then obtained by 5′ and 3′ rapid amplifications of cDNA ends (RACE). The 3204-nt cDNA consists of a 2442-nt coding region, a 553-nt 5′-untranslated region (UTR) and a 209-nt 3′ UTR. It was predicted to encode a polypeptide (813-amino acids) of approximately 90 kDa (Fig. 1A). The expression profiles of the gene were further confirmed by quantitative real-time RT-PCR with the mRNA derived from at least three independent experiments (Fig. 1B). The results showed that the gene was significantly upregulated at 7 day post inoculation (dpi); the expression level of this gene in BaMV-inoculated plants was approximately a 4-fold higher than that in mock-inoculated plants (Fig. 1B).

Blastx alignment analysis of this gene showed a 51% identity and a 63% similarity in amino acid sequence to that of a 75-kDa putative microtubule-associated protein (MAP) of *Arabidopsis* (Weerakoon and Marc, 1999) (Fig. S1). This gene from *N. benthamiana* was predicted to contain a TBC domain in the N-terminal region (Figs. S1 and S2) that is commonly present in Rab-GTPase activation proteins (Rab-GAPs) (Pfeffer and Aivazian, 2004; Segev, 2001), suggesting that this gene may play a role in intracellular membrane trafficking. Additionally, in our analysis the TBC domain also appeared in the 75-kDa *Arabidopsis* putative MAP, indicating the functional similarity between these two proteins (Fig. S1). Since this protein contains the complete TBC domain, we then designated this protein as a *N. benthamiana* RabGTPase-activation protein 1 (NbRabGAP1).

Interestingly, this gene has a long 5′ UTR (553 nts) that contains three short open-reading frames (uORFs) located just upstream of the start codon (Fig. 1C), implying that translational control is involved in the regulation of gene expression (Kozak, 2001). Similar characteristics, including a long 5′ UTR and/or uORFs, have been found in the homologous genes of *Arabidopsis* (the two homologous genes were shown to have the complete 5′ UTR sequence, whereas the 5′ UTR of *AtMAP75* has not been defined yet), rice (*Oryza sativa*), and a hybrid populus (*Populus trichocarpa* × *Populus deltoids*) (Fig. 1C) that were also predicted to contain TBC domains. Overall, these results suggested that the gene with a potential Rab-GAP function from *N. benthamiana* was isolated.

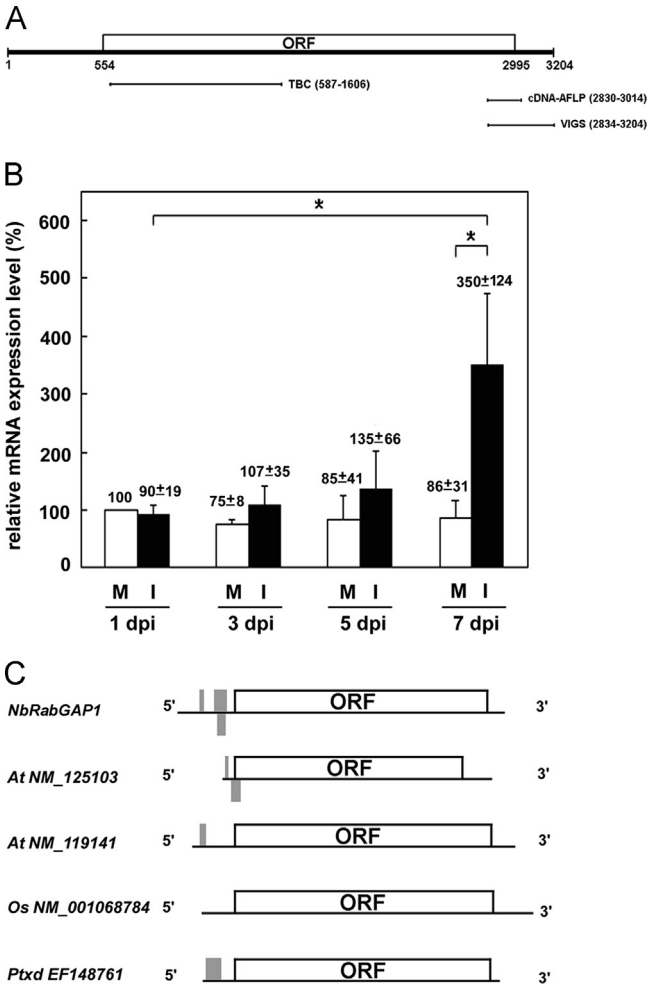

Fig. 1. Illustration of the cDNA organization of *NbRabGAP1* and its expression levels in plants. (A) The full-length cDNA of *NbRabGAP1* with open reading frame (ORF; nt 554–nt 2995) is indicated. The positions of TBC domain (TBC), the DNA fragment cloned from cDNA-AFLP, the DNA fragment used for knocking down the expression of *NbRabGAP1* through VIGS are indicated. (B) The expression levels of *NbRabGAP1* in mock-(M) and BaMV-inoculated (I) plants were measured by qRT-PCR quantification. The measurement in mock-inoculated plants at 1 dpi was set as 100%. The numbers shown above each bar are the average of the levels of *NbRabGAP1* mRNA with the standard error derived from at least three independent experiments. Asterisks indicate statistically significant differences between the indicated group analyzed by the student *T*-test (*$P < 0.05$). (C) Alignment of *NbRabGAP1* cDNA and its homologs from other species. The uORFs are indicated as solid gray boxes. *At*: *Arabidopsis thaliana*; *Os*: *Oryza sativa*; and *Ptxd*: *Populus trichocarpa* × *Populus deltoids*. The GenBank accession numbers are indicated.

Reducing the expression of NbRabGAP1 decreases the accumulation of several RNA viruses in plants

To investigate the possible functions of NbRabGAP1 during BaMV infection, *Tobacco rattle virus* (TRV)-based virus-induced gene silencing (VIGS) system (Ruiz et al., 1998) was used to knock down the expression of *NbRabGAP1* in plants. The cDNA fragment chosen for VIGS (Fig. 1A) contains the entire 3′ UTR and a small portion of the C-terminus coding sequence of NbRabGAP1. We have blasted this cDNA fragment to the draft genome of *N. benthamiana* (Bombarely et al., 2012; Nakasugi et al., 2013) and found only single matched site. We have also used the pssRNAit (Dai and Zhao, 2011) to screen the potential siRNA targets and shown no off-target site of the *N. benthamiana* genome. These results suggest that the knockdown (KD) is specific to target the *NbRabGAP1* gene. The effect on the *NbRabGAP1* reduction was evaluated by measuring the accumulation of viral coat protein.

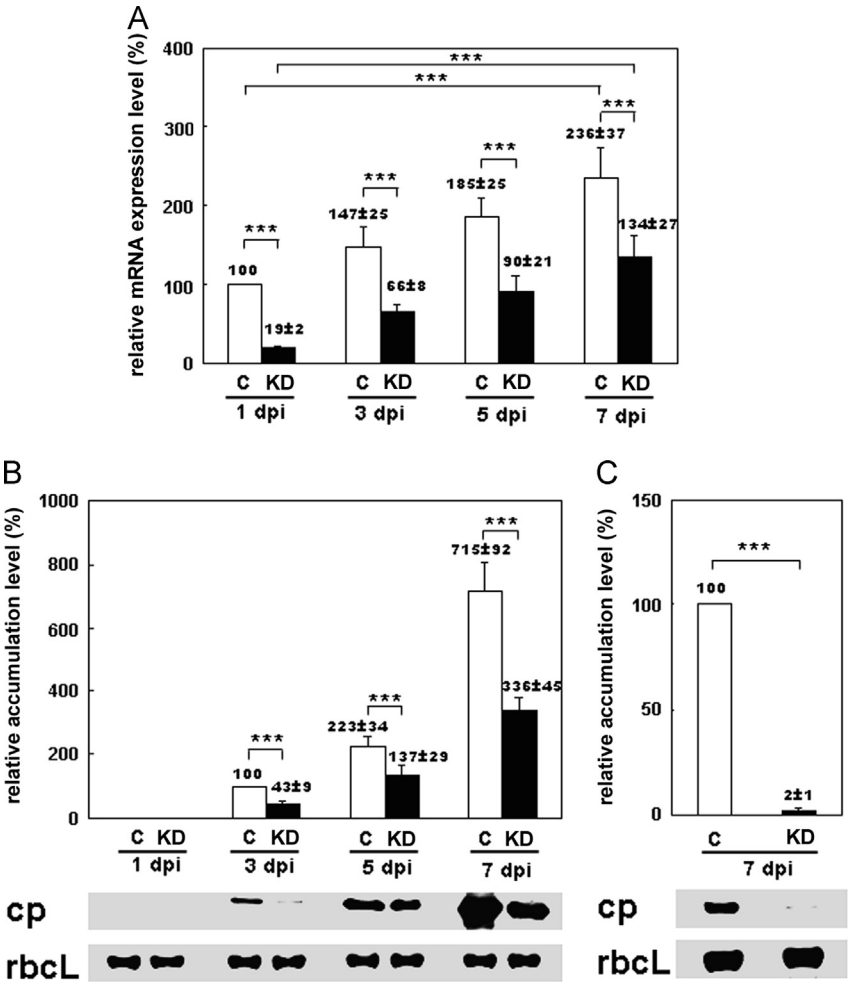

Fig. 2. BaMV coat protein accumulations in *NbRabGAP1*-knockdown (KD) plants. (A) qRT-PCR quantification of *NbRabGAP1* mRNA levels in the inoculated leaves of control (knockdown with pTRV2.GFP) and *NbRabGAP1*-knockdown plants after BaMV infection at the designated dpi. (B) Coat protein accumulation levels detected by Western blotting in the leaves inoculated with viral RNA. The levels in control plants at 3 dpi were set as 100%. (C) Coat protein accumulation level quantified at 7 dpi in the 3rd leaf above the inoculated leaf. The accumulation levels of control plant were set as 100%. Representative results are shown under the statistic results in (B) and (C). The numbers shown above each bar are the average of the relative levels of *NbRabGAP1* mRNA in (A) and BaMV coat protein in (B) and (C) with the standard errors derived from at least three independent experiments. C: *GFP*-knockdown control plants; KD: *NbRabGAP1*-knockdown plants. cp: BaMV coat protein; and rbcL: RuBisCO large subunit (the loading control for normalization). Asterisks indicate statistically significant differences between the indicated group analyzed by the student *T*-test (***$P < 0.001$).

The *NbRabGAP1*-knockdown plants exhibited no morphological difference (Fig. S3) to those of the control plants, with green fluorescent protein gene (GFP) or luciferase gene (Luc) knockdown, suggesting that the reduction of *NbRabGAP1* expression has no significant effect on plant development. After BaMV inoculation, the expression profiles of *NbRabGAP1* in the control and knockdown plants were all upregulated (Fig. 2A). The expression level of *NbRabGAP1* in the knockdown plants was about twofold less than that in the control plants (Fig. 2A). The accumulation levels of the viral coat protein were reduced significantly in the inoculated leaves of the *NbRabGAP1*-knockdown plant compared to those of the control plants (approximately a twofold decrease at 7 dpi, Fig. 2B). The accumulation levels of coat protein in the systemic leaves (the third leaf above the inoculated leaf) were even lower (approximately a 50-fold decrease compared to the control plants, Fig. 2C). The results indicate that the reduction of *NbRabGAP1* expression blocks the spreading of BaMV in plants (both inoculated and systemic leaves).

To examine whether NbRabGAP1 is also involved in the infection of PVX and *Cucumber mosaic virus* (CMV), the control and knockdown plants were each inoculated with both viruses. Similar to BaMV, the expression of *NbRabGAP1* was upregulated approximately twofold compared to that in the control plants at 7 dpi of PVX inoculation (Fig. 3A). However, the expression of *NbRabGAP1* in the CMV-inoculated control plants maintained similarly low levels from day 1 to 7 post inoculation (Fig. 4A). Furthermore, at day 7 the accumulation levels of PVX and CMV coat protein in the *NbRabGAP1*-knockdown plants were reduced approximately 20% and 30%, respectively, compared to those in the control plants (Figs. 3B and 4B). The accumulation levels of viral coat protein in the systemic leaves of the *NbRabGAP1*-knockdown plants were reduced to 67% and 39% that in the control plants for PVX and CMV, respectively (Figs. 3C and 4C). Although the effects on the accumulation levels of PVX and CMV coat proteins in the knockdown plants were not as severe as those of BaMV, the reduction of PVX and CMV did have the statistical significance both in the inoculated and systemic leaves. In summary, these results suggested that *NbRabGAP1* could be involved in a common process by which these plant viruses accumulate.

NbRabGAP1 has a role in cell-to-cell movement of BaMV

To inspect whether the low levels of BaMV accumulation in the *NbRabGAP1*-knockdown plants are due to an effect on the viral RNA replication, BaMV RNA was transfected into the protoplasts derived from the control or the *NbRabGAP1*-knockdown plants.

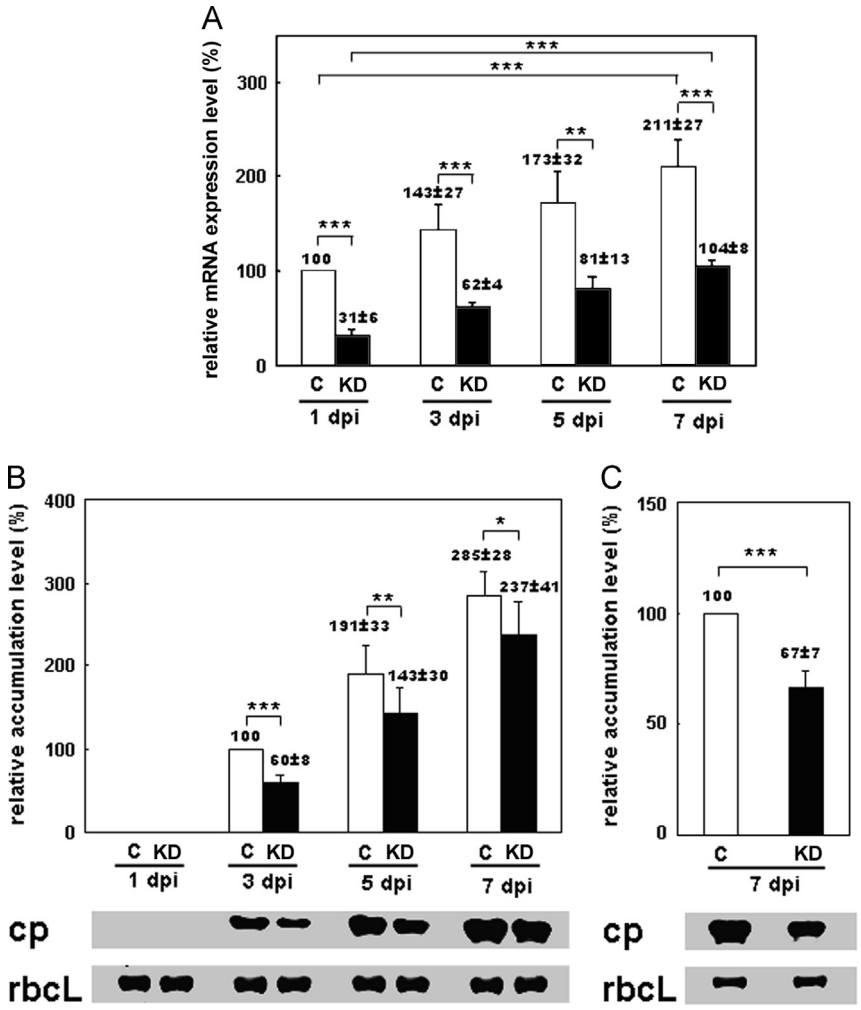

Fig. 3. PVX coat protein accumulations in *NbRabGAP1*-knockdown (KD) plants. (A) qRT-PCR quantification of *NbRabGAP1* mRNA levels in the inoculated leaves of control (knockdown with pTRV2.GFP) and *NbRabGAP1*-knockdown plants after PVX infection. (B) Coat protein accumulation levels detected by Western blotting in viral RNA-inoculated leaves. The coat protein levels in control plants at 3 dpi were set as 100%. (C) Coat protein accumulation levels in the 3rd leaf above the inoculated leaf at 7 dpi. The accumulation levels in control plants were set as 100%. Representative results are shown under the statistic results in (B) and (C). The numbers shown above each bar are the average of the relative levels of *NbRabGAP1* mRNA in (A) and PVX coat protein in (B) and (C) with the standard errors derived from at least three independent experiments. C: *GFP*-knockdown control plants; KD: *NbRabGAP1*-knockdown plants. cp: PVX coat protein; and rbcL: RuBisCO large subunit (the loading control for normalization). Asterisks indicate statistically significant differences between the indicated group analyzed by the student *T*-test ($^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$).

Total RNAs and proteins were extracted from the protoplasts at 24 h and 48 h post-inoculation. The expression levels of *NbRabGAP1* in the knockdown protoplasts were about 10–20% of those in the control protoplasts (Fig. 5A). However, the accumulation levels of BaMV coat protein were similar in both the knockdown and the control protoplasts at 24 h and 48 h post-inoculation (Fig. 5B). Overall these results indicated that the reduction of *NbRabGAP1* expression did not interfere with the viral protein accumulation in protoplasts but it did affect those in the inoculated and systemic leaves. These observations suggest that NbRabGAP1 is involved in the movement of BaMV.

To test the hypothesis that NbRabGAP1 is involved in assisting the cell-to-cell movement of BaMV, the area of viral infection foci on the inoculated leaves was measured by fluorescent microscopy. The plasmid pCBG (containing a 35S promoter driven BaMV infectious cDNA could express the GFP under the control of a BaMV subgenomic promoter) was used to inoculate the control (pTRV2. Luc containing luciferase gene fragment) and the *NbRabGAP1*-knockdown plants. The area of the resultant green fluorescent foci on the inoculated leaves were measured at 4 dpi (Fig. 6A). A total number of 25 and 24 foci from the control and the *NbRabGAP1*-knockdown plants were measured, respectively. On average, the foci in the control plants (mean area = 3.8 mm^2) were larger than those in the *NbRabGAP1*-knockdown plants (mean area = 2.2 mm^2; Fig. 6B) with the statistical significance. Together with our previous observations that the coat protein accumulation of BaMV was reduced in the inoculated leaves of the *NbRabGAP1*-knockdown plants but not in the knockdown protoplasts, these results indicated that the cell-to-cell movement of BaMV was restricted when the expression of *NbRabGAP1* was decreased.

Transiently expressed NbRabGAP1 but not the catalytic site mutant can help the accumulation of BaMV

To confirm NbRabGAP1 plays a positive role in helping the spreading of BaMV in *N. benthamiana* plants, we transiently expressed an Orange fluorescent protein (OFP)-fused NbRabGAP1 (NbRabGAP1-OFP, approximately 120 kDa) after two days of virus inoculation (Fig. 7A). Furthermore, we have also transiently expressed a substitution mutant (NbRabGAP1(R111A)-OFP; mutation at the active site of TBC domain of the GAP) which failed to activate the GTP hydrolysis activity of Rab GTPase (Pan et al., 2006). The results showed that the accumulation levels of BaMV coat protein was significantly increased to 163% of that of the

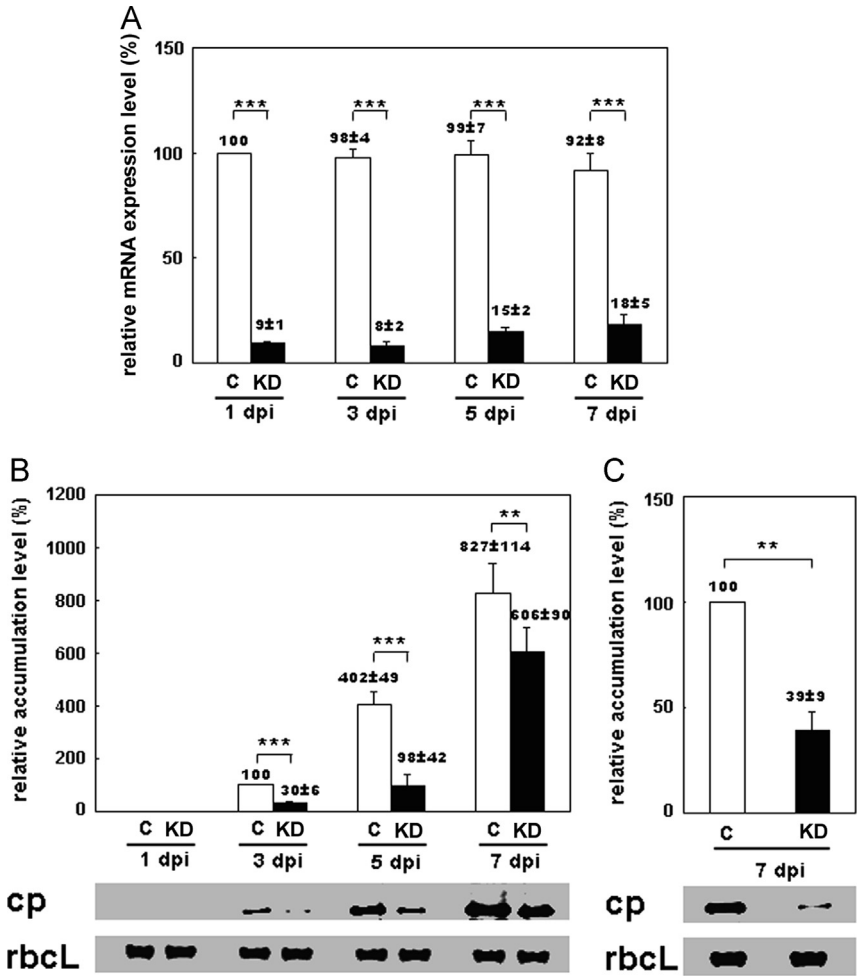

Fig. 4. CMV coat protein accumulations in *NbRabGAP1*-knockdown (KD) plants. (A) qRT-PCR quantification of *NbRabGAP1* mRNA levels in control (knockdown with pTRV2. GFP) and *NbRabGAP1*-knockdown plants after CMV infection. The mRNAs were isolated from the CMV-inoculated leaves. (B) The coat protein accumulation levels detected by Western blotting in viral RNA-inoculated leaves. The coat protein levels in control plants at 3 dpi were set as 100%. (C) Coat protein accumulation levels in the 3rd leaf above the inoculated leaf were quantified at 7 dpi. The accumulation levels in control plant were set as 100%. Representative results are shown under the statistic results in (B) and (C). The numbers shown above each bar are the average of the relative levels of *NbRabGAP1* mRNA in (A) and CMV coat protein in (B) and (C) with the standard errors derived from at least three independent experiments. C: *GFP*-knockdown control plants; KD: *NbRabGAP1*-knockdown plants. cp: CMV coat protein; and rbcL: RuBisCO large subunit, (the loading control for normalization). Asterisks indicate statistically significant differences between the indicated group analyzed by the student T-test ($^{**}P < 0.01$ and $^{***}P < 0.001$).

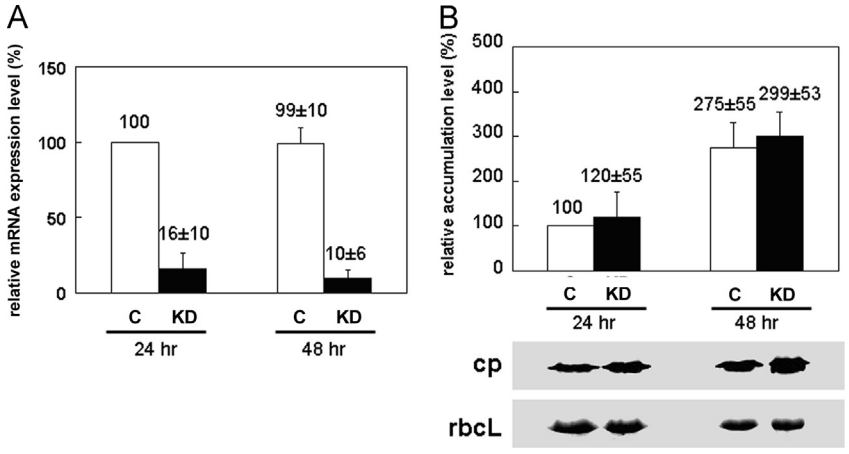

Fig. 5. Coat protein accumulation levels detected in the protoplasts derived from control and knockdown (KD) plants. (A) qRT-PCR quantification of *NbRabGAP1* mRNA levels in the control (*GFP*-knockdown) and *NbRabGAP1*-knockdown protoplasts after BaMV inoculation. (B) Coat protein accumulation levels detected by Western blotting in viral RNA-inoculated protoplasts. The accumulation levels in the control protoplasts at the 24 h post-transfection were set as 100%. Representative results are shown under the statistical results in (B). The numbers shown above each bar are the average of the relative levels of *NbRabGAP1* mRNA in (A) and BaMV coat protein in (B) with the standard errors derived from at least three independent experiments. C: protoplasts derived from *GFP*-knockdown plants; KD: protoplasts derived from *NbRabGAP1*-knockdown plants. cp: BaMV coat protein; and rbcL: RuBisCO large subunit (the loading control for normalization).

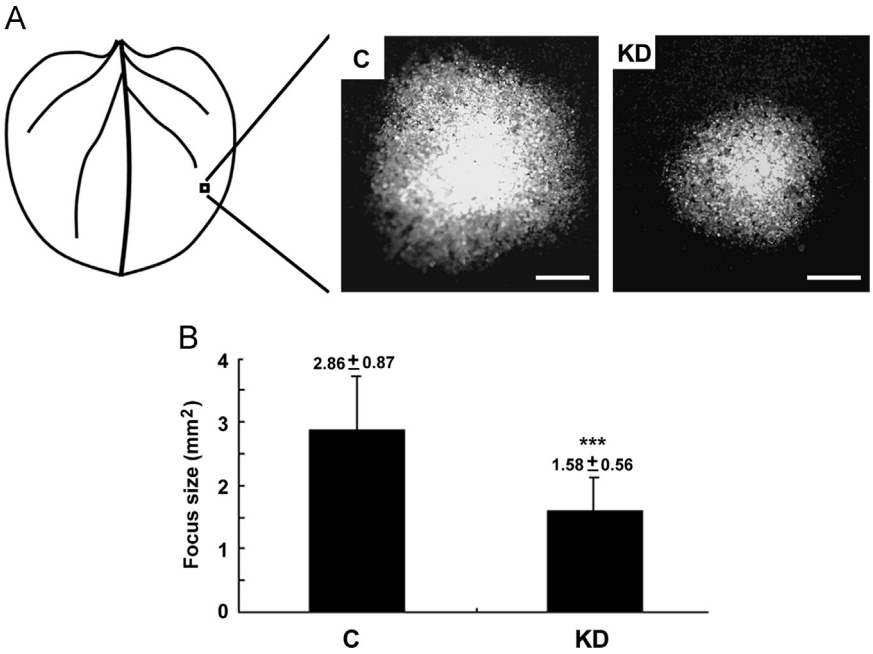

Fig. 6. The effects of the *NbRabGAP1*-knockdown on BaMV infection. (A) The areas of the GFP fluorescent foci in the inoculated leaves of the *Luc*-knockdown control (C, knockdown with the luciferase cDNA fragment) and *NbRabGAP1*-knockdown (KD) plants were measured under the fluorescent microscope after the plants were inoculation with the pCBG plasmids. Bar length = 0.5 mm. (B) Statistical analysis of the results obtained in (A). *X*-axis is the GFP focus size (mm^2). The numbers shown above the statistic bar were the average and the standard deviation of 25 and 24 foci from C and *NbRabGAP1*-knockdown plants, respectively.

control plants when NbRabGAP1 was expressed at three days post-infiltration. However, the coat protein accumulation was increased to 120% of that of the control plants but statistically insignificant when mutant NbRabGAP1(R111A) was expressed (Fig. 7B). The results suggest that the single point mutation at the predicted catalytic site NbRabGAP1(R111A) might not completely block the function of helping the movement of BaMV. However, the fully GAP function of NbRabGAP1 is necessary to entirely support the spreading of BaMV.

Discussion

The reduction of BaMV coat protein accumulation in the inoculated and systemic leaves but not in protoplasts of the *NbRabGAP1*-knockdown plants suggested that NbRabGAP1 could be important for virus movement (Figs. 2, 5 and 6). The enhancement of BaMV accumulation in the *NbRabGAP1*-expressed plants supported the idea of a positive role of NbRabGAP1 in helping BaMV spread (Fig. 7). This role could be applied to other viruses such as PVX and CMV (Figs. 3 and 4). However, the induction of *NbRabGAP1* expression occurred after infection with potexviruses (BaMV and PVX) but not with CMV (Fig. 4A). It is possible that the requirement of NbRabGAP1 may have differences among the infection cycles of different viruses. Further, the upregulation of *NbRabGAP1* after BaMV and PVX infection is not likely to be involved in the host's defense response; it is more likely that a system is utilized by the viruses. Within the context of intracellular movement regulation, NbRabGAP1 possibly functions by facilitating potexviral spreading between cells.

NbRabGAP1, predicted to be a Rab-GAP, can assist the recycling of Rabs to allow Rabs to engage in another round of membrane-vesicle budding, movement, docking, or fusion. It is possible that NbRabGAP1 helps to target the vRNP and/or TGB proteins (in potexviruses) to PD via the endomembrane secretory or some other specific pathway (most likely to be the ER and post-ER secretory pathways) (Ju et al., 2005; Tilsner et al., 2012; Tseng et al., 2009; Yoshimoto et al., 2010). The viral RNA replicating in the infected cells in association with the endomembrane systems is a common strategy for most of the plant positive-sense RNA viruses (Pena and Heinlein, 2012; Tilsner et al., 2012). To accomplish the successful infection, the newly synthesized progeny RNAs has to move from the replication site to neighboring cells through the secretory pathway. In the case of PVX, the TGBp1 forms the core of X-bodies containing vRNAs, other movement-associated proteins and virions that are proposed to link viral RNA replication and movement and possibly the encapsidation (Tilsner et al., 2012). The TGBp2 and TGBp3 are the ER-associated transmembrane proteins and the TGBp2 induces the ER-derived granular vesicles (Ju et al., 2005; Toyooka et al., 2006). TGBp2 mutant failed to induce the vesicle formation was shown to fail in cell-to-cell trafficking (Yoshimoto et al., 2010). Lately, the movement protein of *Chinese wheat mosaic virus* was demonstrated to form the ER-derived vesicular structure and could complement the cell-to-cell movement-defective PVX (Qin et al., 2007). The possible role of NbRabGAP1 is involved in helping the viral movement proteins to induce these vesicles. Alternatively, NbRabGAP1 might regulate the return of TGB proteins from PD via the endocytotic pathway as those found in PMTV (Haupt et al., 2005). However, this observation has not been reported yet in the potexviruses.

Materials and methods

Plant and virus

The growth condition for *N. benthamiana* was 16/8 h of light/dark at 28 °C. BaMV strain S (Lin and Hsu, 1994), PVX strain Taiwan, and CMV strain NT9 (Hsu et al., 1995) were used as the infection agents.

Quantitative RT-PCR

The reverse transcription reactions were carried out with the Powerscript reverse transcriptase (BD Biosciences, San Jose, CA, USA). For the SYBR Green I-based quantitative RT-PCR, two sets of primers were used in quantifying the *NbRabGAP1* mRNA expression levels

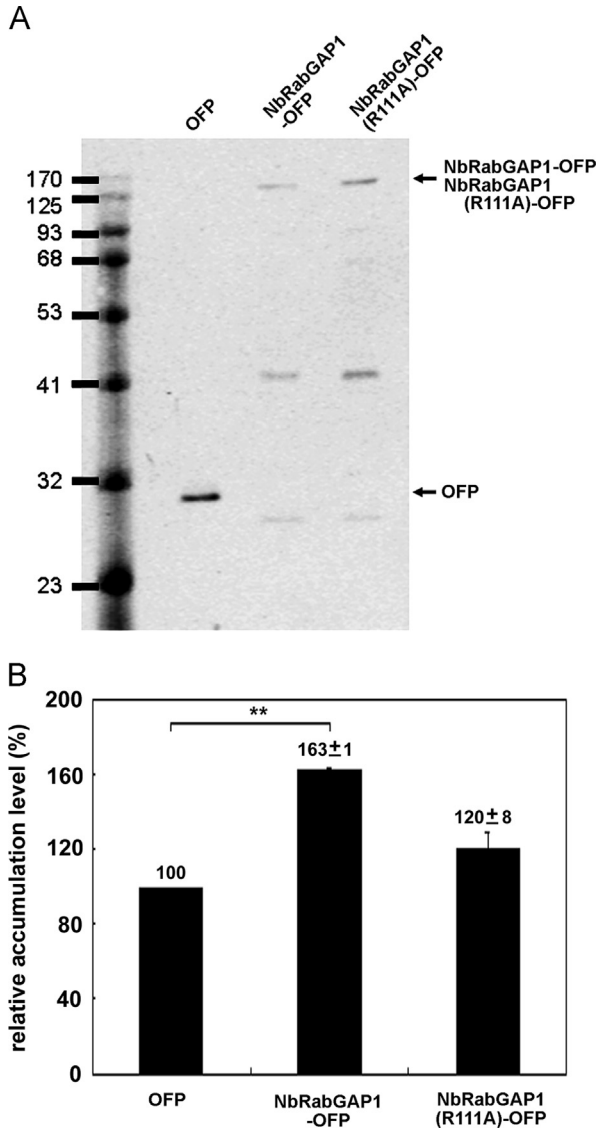

Fig. 7. The effects of the expression of *NbRabGAP1*on BaMV infection. (A) The Western blotting analysis of the transiently expressed NbRabGAP1-OFP, mutant NbRabGAP1(R111A)-OFP and the OFP indicated on the top of each lane by agro-infiltration on the *N. benthamiana* leaves. The total proteins were extracted and separated onto a 12% SDS-polyacrylamide gel. After transferring to the membrane, the proteins were detected with the antibody against OFP. (B) The relative accumulation of BaMV coat protein was detected at 5 days post inoculation (dpi) on the inoculated leaves while NbRabGAP1-OFP, mutant NbRabGAP1(R111A)-OFP, or OFP only (as a control) was transiently expressed on the same leaves at 3 dpi. The data measured from western blots were normalized with the large subunit of RuBisCO (rbcL). The numbers shown above the statistic bar were the average and the standard error of at least three independent experiments. Asterisks indicate statistically significant differences between the indicated group analyzed by the student T-test ($^{**}P < 0.01$ and $^{***}P < 0.001$).

(Fig. 1A). Primer pair 1, 5′GAATATCCAGGTGATTGA3′ and 5′GAGATATGAGAAGCCGA3′, was used to confirm the cDNA-AFLP differentially expressed pattern; and primer pair 2, 5′GAAAGTGT CGACTCTGGG3′ and 5′TACAGATTCTCCCTTGCTAA3′, was used for the VIGS experiments. Primers for the actin mRNA which was included as the internal control for the normalization of *NbRabGAP1* were 5′GTGGTTTCATGAATGCCAGCA3′ and 5′GATGAAGATACTCACAGAAAGA3′.

cDNA cloning by RACE

RACE reactions were carried out to clone the full-length *NbRabGAP1* cDNA. For the 3′ RACE, the reverse transcription reaction driven by the Powerscript reverse transcriptase included primers 5′GCCCCGGGATCCT$_{20}$3′ and 5′GAGAATATCCAG GTGATTGA3′. The 5′RACE was conducted using the BD SMART™ RACE cDNA Amplification Kit (BD Biosciences, San Jose, CA, USA) according to the instructions provided by the manufacturer. The gene-specific primer was 5′ CCGGGTACATGCCGAAATTTTGAATC3′. Both RACE products were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced.

Constructs for transient expression

The PCR primer pairs, GAP1-5′ (5′GGGATGGCTGCAATTGCAATTGAG3′) and GAP1-3′ (5′GGGTCATTACATCTCCGAGAGGAGG3′), are used for NbRabGAP1 fluorescent protein fusion construct; GAP1mu-5′ (5′CTCAGGATATAAAGCCGAA3′) and GAP1-3′ are used for mutagenesis on the TBC domain of NbRabGAP1 that changes the catalytic residue arginine (R) to alanine (A). The PCR products were cloned and sequenced. The full-length of NbRabGAP1 and NbRabGAP1(R111A) were then subcloned into the orange fluorescent protein (OFP) containing vector pBin-OFP driven by the *Cauliflower mosaic virus* 35S promoter (reconstructed from pmKO2-S1; MBL international, Woburn, USA), the resultant constructs were designated as NbRabGAP1-OFP and NbRabGAP1 (R111A)-OFP. *Agrobacterium* containing the plasmid encodes NbRabGAP1-OFP, NbRabGAP1(R111A)-OFP or pBIN61-HcPro was cultured to OD600 = 1 and induced with 500 μM acetosyringone in 10 mM $MgCl_2$. Each construct in the *Agrobacterium* broth was mixed with pBIN61-HcPro broth in a 1:1 volume ratio and infiltrated into the *N. benthamiana* leaves.

Virus-induced gene silencing

The silencing system was constructed in the TRV vector. A 371-bp *NbRabGAP1* cDNA fragment (nt 2834–3204, including the entire 3′ UTR), amplified by using the primer pair 5′GAGAATATCCAGGTGATTGA3′ and 5′GCTAACAACATATACTGTTACA3′, was cloned into the pTRV2 vector with *Sma*I site. The resulted construct was designated as pTRV2.RabGAP1. Two control plasmids, pTRV2.GFP and pTRV2.Luc, containing portions of GFP and Luciferase gene were constructed. Plasmids pTRV2.RabGAP1, pTRV2.GFP and pTRV2.Luc were transformed into the *Agrobacterium tumefaciens* C58C1 strain by electroporation.

To knock down *NbRabGAP1* expression in *N. benthamiana*, the *A. tumefaciens* C58C1 containing pTRV1, pTRV2.GFP, pTRV2.Luc or pTRV2.RabGAP1 was cultured to OD_{600} = 1 at 30 °C before induction by the addition of 130 μM acetosyringone in 10 mM $MgCl_2$ for 3 h at room temperature. Subsequently, the pTRV2.GFP-, pTRV2.Luc- or pTRV2.RabGAP1-containing *A. tumefaciens* C58C1 was mixed with pTRV1-containing *A. tumefaciens* C58C1 at a 1:1 volume ratio. The 1st and 2nd leaves were infiltrated with the mixed broth at the four-leaf stage (seedlings with two cotyledons and two leaves) and 500 ng of BaMV, PVX, or CMV virion RNA was inoculated onto the 6th leaf when it was mature. Total RNAs and proteins were extracted from the leaves at 1, 3, 5, and 7 dpi and measured for the *NbRabGAP1* mRNA and viral coat protein levels, respectively. For the protoplast inoculation assay, protoplasts prepared from the 6th leaf were transfected with 1 μg of BaMV genomic RNA. The levels of *NbRabGAP1* mRNA and viral coat proteins were measured at 24 and 48 h of post-inoculation.

Detection of cell-to-cell movement

The viral cell-to-cell movement efficiency was determined by the fluorescence distribution of virus-encoded GFP. The 6th leaf of the VIGS plants was inoculated with 5 μg of the pCBG plasmid, a GFP-expression BaMV viral vector (Lin et al., 2004). GFP fluorescent focus

was identified under an Olympus IX71 inverted fluorescent microscope and the images were processed by Adobe Photoshop CS. Due to the irregular circumference of the foci, the green fluorescent area of each focus was calculated through the Image J software (http://rsbweb.nih.gov/ij/).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.09.021.

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