Short title: Role of NbRPL1 in TVBMV infection

*Corresponding Author: Xiang-Dong Li (xdongli@sdau.edu.cn)

Title: The chloroplast ribosomal protein large subunit 1 interacts with viral polymerase and promotes virus infection

Authors: De-Jie Cheng, Xiao-Jie Xu, Zhi-Yong Yan, Carlos Kwesi Tettey, Le Fang, Guang-Ling Yang, Chao Geng, Yan-Ping Tian†, Xiang-Dong Li*

Affiliations: Shandong Provincial Key Laboratory of Agricultural Microbiology, Laboratory of Plant Virology, Department of Plant Pathology, College of Plant Protection, Shandong Agricultural University, Tai’an, Shandong 271018, China

† Senior author

One-sentence summary: A chloroplast ribosomal protein competes with a host degradation factor to bind the viral RNA-dependent RNA polymerase and thus promotes tobacco vein banding mosaic virus infection.

Author Contributions:

DC, YT, and XL designed the experiments; DC, XX, and ZY performed the experiments; DC, CT, GY, and XL analyzed the data; GC and LF provided the study materials; DC, YT, and XL wrote the manuscript.

ORCID IDs: 0000-0001-9838-0045 (X.-D. L.); 0000-0002-3452-2013 (Y.-P. T.);
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Chloroplasts play an indispensable role in the arms race between plant viruses and hosts. Chloroplast proteins are often recruited by plant viruses to support viral replication and movement. However, the mechanism by which chloroplast proteins regulate potyvirus infection remains largely unknown. In this study, we observed that *Nicotiana benthamiana* ribosomal protein large subunit 1 (NbRPL1), a chloroplast ribosomal protein, localized to the chloroplasts via its N-terminal 61 amino acids (transit peptide), and interacted with tobacco vein banding mosaic virus (TVBMV) nuclear inclusion protein b (NIb), an RNA-dependent RNA polymerase. Upon TVBMV infection, NbRPL1 was recruited into the 6K2-induced viral replication complexes in chloroplasts. Silencing of *NbRPL1* expression reduced TVBMV replication. NbRPL1 competed with NbBeclin1 to bind NIb, and reduced the NbBeclin1-mediated degradation of NIb. Therefore, our results suggest that NbRPL1 interacts with NIb in the chloroplasts, reduces NbBeclin1-mediated NIb degradation, and enhances TVBMV infection.

**Keywords:** chloroplast ribosomal proteins, chloroplast targeting, chloroplast transit peptide, NbBeclin1, RNA-dependent RNA polymerase, *Tobacco vein banding mosaic virus*, virus replication complex.
INTRODUCTION

Chloroplasts are organelles that conduct photosynthesis by trapping energy from sunlight, and are the site of synthesis for key biochemical components. Chloroplasts are also targets of plant viruses (Li et al., 2016; Zhao et al., 2016; Bhattacharyya and Chakraborty, 2018). Viral infections cause a substantial reduction in photosynthesis (Montasser et al., 2012; Bhattacharyya et al., 2015; Li et al., 2016; Zhao et al., 2016), and expression of chloroplast-related genes is affected during viral infection (Li et al., 2016). Chlorosis in Nicotiana benthamiana leaves caused by the Rice stripe virus is due to the downregulation of chloroplast-related gene expression (Shi et al., 2016). Our previous study showed that Tobacco vein banding mosaic virus (TVBMV) infection causes differential expression of many chloroplast-related genes in N. benthamiana (Geng et al., 2017).

Chloroplast proteins are involved in several viral infection processes, including replication and movement. The N. benthamiana chloroplast protein glyceraldehyde 3-phosphate dehydrogenase subunit A (NbGAPDH-A) interacts with the movement protein (MP) of Red clover necrotic mosaic virus (RCNMV), and silencing of NbGAPDH-A expression inhibits MP localization to cortical viral replication complexes (VRCs) and chloroplasts (Kaido et al., 2014). Chloroplast phosphoglycerate kinase (chl-PGK) is used by the Bamboo mosaic virus (BaMV) RNA to target chloroplasts; knocking down the expression levels of chl-PGK reduced BaMV coat protein accumulation (Cheng et al., 2013). The N. benthamiana Rubisco small subunit (NbRbCS) interacts with the MP of Tomato mosaic virus; silencing of NbRbCS expression was reported to delay the appearance of systemic symptoms (Zhao et al., 2013). The expression levels of the chloroplast ATP-synthase γ-subunit (AtpC) and Rubisco activase (RCA) are reduced in Tobacco mosaic virus (TMV)-infected N. benthamiana plants, and silencing either gene enhanced viral accumulation (Bhat et al., 2013).
Potyviruses (genus *Potyvirus*, family *Potyviridae*) form the largest plant RNA virus group, and cause substantial economic losses to crop production worldwide (Ivanov et al., 2014). The potyvirus genome is a positive-sense single-stranded RNA that encodes 11 proteins (Chung et al., 2008; Revers and García, 2015). Among these viral proteins, nuclear inclusion protein b (Niib) is an RNA-dependent RNA polymerase (RdRp) that contains a conserved GDD motif (Koonin, 1991). A previous study has shown that the light-induced protein (LIP) localizes in chloroplasts and interacts with *Wheat yellow mosaic virus* (WYMV) Niib to facilitate viral infection (Zhang et al., 2019). Cytoplasmic ribosomal protein P0 is present in the ribonucleoprotein-complexes that can be immunoprecipitated using *Potato virus A* (PVA) Niib and promotes PVA infection (Hafrén et al., 2013). However, no chloroplast ribosomal protein has been reported to interact with Niib.

In this study, we determined that the *N. benthamiana* chloroplast ribosomal protein larger subunit 1 (NbRPL1) can interact with TVBMV Niib. Further, we studied the function of NbRPL1 in the infection of TVBMV.

**RESULTS**

**TVBMV Niib interacts with chloroplast protein NbRPL1**

Interaction between chloroplast proteins and TVBMV Niib was determined after extracting total protein from *N. benthamiana* leaves transiently expressing MYC:Niib. Total protein was immunoprecipitated with anti-MYC agarose beads, and separated by SDS-PAGE. The co-precipitated proteins in the gel were identified by liquid chromatography tandem mass spectrometry (LC-MS/MS) followed by database searches. Two chloroplast proteins, the 50S ribosomal protein large subunit 1 (NbRPL1) and the elongation factor Tu (NbEF-Tu) of *N. benthamiana*, were selected for further analyses due to their high confidence and peptide coverage (Supplemental Figure S1).
Pull-down and bimolecular fluorescence complementation (BiFC) assays were then conducted to investigate whether NbRPL1 or NbEF-Tu interacted directly with TVBMV NlB. HIS:eGFP:NbRPL1 and HIS:MBP:MYC:NlB, HIS:eGFP:NbEF-Tu and HIS:MBP:MYC:NlB, and HIS:eGFP and HIS:MBP:MYC:NlB were incubated with GFP-Trap_A beads, respectively, followed by western blotting. Results showed that TVBMV NlB interacted directly with NbRPL1, but not with NbEF-Tu (Figure 1A). To further verify the interaction between NlB and NbRPL1, we transiently co-expressed NlB:YN and NbRPL1:YC, NlB:YN and YC, or YN and NbRPL1:YC in *N. benthamiana* leaves. Confocal microscopy revealed YFP fluorescence in the chloroplasts in *N. benthamiana* leaf cells co-expressing NlB:YN and NbRPL1:YC (Figure 1B). Both pull-down and BiFC assays showed that TVBMV NlB interacted with NbRPL1 directly. Transient expression of NbRPL1:DsRed or NbRPL1:eGFP in *N. benthamiana* leaves revealed that NbRPL1 was localized in chloroplasts (Figure 1C).

**Chloroplast transit peptide (cTP) domain is required for NbRPL1 chloroplast targeting**

The cTP plays an important role in targeting proteins to chloroplasts (Bionda et al., 2010). We predicted the potential function of NbRPL1 cTP using the ChloroP 1.1 server (http://www.cbs.dtu.dk/services/ChloroP/) (Emanuelsson et al., 1999). The result showed that the N-terminal 61 amino acids (aa) could form the cTP of NbRPL1, with the cleavage-site motif of Val-Val-Ala↓Ala (Figure 2A). To verify the cleavage activity of NbRPL1 in *vivo*, we expressed NbRPL1:DsRed in healthy and TVBMV-infected *N. benthamiana* plants. Total protein extracts were incubated with anti-mCherry beads, followed by SDS-PAGE. Two bands of about 64.1 kDa and 57.7 kDa, corresponding to the size of NbRPL1:DsRed with or without cTP (NbRPL1:DsRed and NbRPL1ΔcTP:DsRed), respectively, were observed in both treatments (Figure 2B).

Mapping of the key region in cTP that regulates NbRPL1 chloroplast targeting was performed through transient expression of NbRPL1ΔcTP(N61aa):DsRed, NbRPL1ΔN41aa:DsRed, NbRPL1ΔN20aa:DsRed, NbRPL1ΔN42-61aa:DsRed, NbRPL1ΔN21-61aa:DsRed, or NbRPL1ΔN21-41aa:DsRed in *N. benthamiana* leaves. Confocal microscopy revealed that none of
the truncated mutants were located in chloroplasts, indicating that the integrity of cTP is crucial for NbRPL1 chloroplast targeting (Figure 2C, D).

We also investigated the role of the C-terminal aa in NbRPL1 chloroplast targeting. Transient expression of the truncated mutant NbRPL1Δ1-280aa:DsRed (expressing C-terminal 60 aa) or NbRPL1Δ281-340aa:DsRed (expressing N-terminal 280 aa) in N. benthamiana leaves showed that NbRPL1Δ281-340aa:DsRed was localized in chloroplasts, whereas NbRPL1Δ1-280aa:DsRed was localized in cytoplasm, indicating that the C-terminal aa was dispensable for NbRPL1 chloroplast targeting (Figure 2C, E).

We then co-expressed Nl:b:YN and NbRPL1AcTP:YC, or YN and NbRPL1AcTP:YC in N. benthamiana leaves to investigate whether cTP is required for the interaction between NbRPL1 and Nl:b. The result showed that Nl:b:YN interacted with NbRPL1AcTP:YC (Figure 2F), indicating that cTP is dispensable for the interaction between Nl:b and NbRPL1.

The NbRPL1/Nl:b complex co-localizes with the 6K2-associated VRCs in TVBMV-infected cells

It was worth investigating whether NbRPL1 was also present in the TVBMV VRCs. We transiently co-expressed Nl:b:YN, NbRPL1:YC, and 6K2:DsRed in N. benthamiana leaves, and found that NbRPL1 and Nl:b co-localized in the 6K2-induced vesicles (Figure 3A). We also transiently co-expressed NbRPL1:YC and Nl:b:YN with the nuclear marker H2B:mCherry or the plasmodesmata marker, Arabidopsis thaliana plasmodesmata-localized protein 1 (AtPDLP1), AtPDLP1:DsRed in N. benthamiana leaves. The result showed that the Nl:b/NbRPL1 complex was not localized in the nucleus or plasmodesmata (Figure 3B). Further, in the presence of TVBMV infection, Nl:b and NbRPL1 co-localized with the 6K2-induced VRCs in chloroplasts (Figure 3C).

Silencing of NbRPL1 expression reduces TVBMV systemic movement and replication

The role of NbRPL1 in TVBMV infection in plants was investigated through silencing NbRPL1 expression in N. benthamiana using a TRV-based virus-induced gene silencing
(VIGS) vector, and subsequently inoculating these NbRPL1-silenced plants with TVBMV-GFP. The NbRPL1-silenced N. benthamiana plants showed etiolation of systemic leaves by 8 days post virus inoculation through agroinfiltration (Figure 4A). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis showed that at 8 days post agroinfiltration (dpai), the accumulation of NbRPL1 mRNA in the NbRPL1-silenced N. benthamiana plants was approximately 10% of that in the control plants (Figure 4B). At 6 days post-TVBMV-GFP inoculation, UV illumination induced green fluorescence in the systemic leaves of the TRV-GUS-treated (control) plants, but not in the NbRPL1-silenced plants (Figure 4C). Western blotting result showed that TVBMV coat protein (CP) had accumulated in the systemic leaves of the control plants, but not in the systemic leaves of the NbRPL1-silenced plants (Figure 4D). Reverse transcription semi-quantitative PCR (RT-sqPCR) result showed that viral RNA was present in the systemic leaves of the control plants, but not in the systemic leaves of the NbRPL1-silenced plants (Figure 4E). These results suggest that silencing NbRPL1 abolished TVBMV long-distance movement.

The effect of NbRPL1 silencing on TVBMV accumulation and replication in the inoculated leaves was determined through inoculation of TVBMV-GFP to NbRPL1-silenced and non-silenced N. benthamiana leaves via agroinfiltration. At 72 h post agroinfiltration (hpai), the TVBMV-GFP-inoculated control leaves showed stronger green fluorescence than the TVBMV-GFP-inoculated NbRPL1-silenced leaves (Figure 4F). Western blotting analysis indicated that TVBMV CP accumulation was reduced in the NbRPL1-silenced N. benthamiana leaves (Figure 4G). RT-sqPCR showed that the accumulation level of TVBMV-GFP viral RNA was reduced in the inoculated leaves of the NbRPL1-silenced plants (Figure 4H). We also inoculated the NbRPL1-silenced and non-silenced control N. benthamiana leaves with TVBMVΔP3NPIPO-GFP, a mutant defective for cell-to-cell movement, to exclude the effect of intracellular viral movement on RNA accumulation. The RT-qPCR assays showed that the level of TVBMVΔP3NPIPO-GFP viral RNA in the leaves of the NbRPL1-silenced plants was significantly reduced compared to the non-silenced control plant by 36 hpai (Figure 4I).
The effect of NbRPL1 on the subcellular localization of 6K2-induced vesicles was investigated by transient expression of 6K2:DsRed in NbRPL1-silenced leaves. Confocal microscopy showed that 6K2:DsRed was localized in the chloroplasts of the NbRPL1-silenced and control plants (Figure 4J). To investigate whether silencing of NbRPL1 expression affects the recruitment of Niib to VRC in TVBMV infection, we co-expressed Niib:mCherry and TVBMV-6K2:GFP in the leaves of the NbRPL1-silenced and control plants. We found that Niib:mCherry co-localized with 6K2 in the chloroplasts in control leaves. However, the co-localization of Niib and VRC was reduced in the NbRPL1-silenced plant leaves (Figure 4K). The result showed that silencing of NbRPL1 expression did not affect the localization of 6K2:DsRed in chloroplasts, but reduced the co-localization of Niib and VRCs in chloroplasts during TVBMV infection.

Overexpression of NbRPL1 increases the accumulation of TVBMV Niib and RNA

The effect of NbRPL1 overexpression on TVBMV infection was determined through agroinfiltration of TVBMV-GFP into NbRPL1-overexpressing N. benthamiana leaves. At 96 hpai, representative TVBMV-GFP infection foci in the infiltrated leaves were photographed under a stereo fluorescence microscope (Figure 5A). The mean number of TVBMV-GFP-infected cells in each infection site in the NbRPL1-overexpressed leaves was 47±20, whereas that in the control leaves was 31±14 (Figure 5A, B), indicating that overexpression of NbRPL1 in N. benthamiana leaves enhanced virus cell-to-cell movement. We also agroinfiltrated the leaves of the NbRPL1-overexpression and control plants with TVBMV\textsuperscript{AGDD}-RLUC/FLUC. Results of the dual-luciferase activity assay showed that, by 60 hpai, the activities of RLUC and FLUC from TVBMV\textsuperscript{AGDD}-RLUC/FLUC (Supplemental Figure S2) in the NbRPL1-overexpression N. benthamiana leaves was similar to that in the leaves of the control plants (Figure 5C), indicating that overexpression of NbRPL1 did not affect the translation efficiency.

TVBMV-GFP was transfected into the protoplasts isolated from the NbRPL1-overexpression or control N. benthamiana leaves to investigate the effect of NbRPL1 on TVBMV replication. Viral RNA accumulation was detected through RT-qPCR at 12 h post transfection. The results showed that TVBMV RNA accumulation in the NbRPL1-overexpression protoplasts was significantly higher than that in the control
protoplasts (Figure 5D). We then agroinfiltrated TVBMV\textsuperscript{ΔP3NPIPO}-GFP into the NbRPL1-overexpression and control \textit{N. benthamiana} leaves. RT-qPCR results showed that, by 36 hpai, TVBMV RNA accumulation in the NbRPL1-overexpressing leaves was significantly higher than that in the control (Figure 5E), indicating that NbRPL1 could enhance TVBMV replication.

The effect of NbRPL1 on TVBMV NIb accumulation was determined through co-expressing NbRPL1:DsRed and MYC:NIb in the TVBMV-infected \textit{N. benthamiana} leaves. Western blotting result showed that NIb accumulation in the NbRPL1-overexpressing \textit{N. benthamiana} leaves was higher than that in the control \textit{N. benthamiana} leaves (Figure 5F).

These results showed that overexpression of NbRPL1 promoted the accumulation of NIb and viral RNA, which promoted the cell-to-cell movement of TVBMV.

\textbf{NbRPL1 interferes with the binding and degradation of NIb by NbBeclin1}

Eight autophagy-related genes (ATGs): viz., \textit{NbATG2}, \textit{NbATG3}, \textit{NbATG4}, \textit{NbATG8a}, \textit{NbATG8c}, \textit{NbATG8i}, \textit{NbBeclin1}, and \textit{NbVPS15}, were upregulated in the TVBMV-infected plants at 7 and 16 dpi, as indicated by RT-qPCR analysis (Figure S3A, B), suggesting that TVBMV infection could activate the autophagy pathway. To verify whether NbBeclin1 could recognize TVBMV NIb, we performed co-immunoprecipitation (Co-IP), yeast two-hybrid (Y2H), and BiFC assays. The results showed that NbBeclin1 interacted with TVBMV NIb in \textit{vitro} and \textit{in vivo} (Figure 6A–C). NbBeclin1:eGFP and MYC:NIb were co-expressed in \textit{N. benthamiana} leaves to ascertain whether NbBeclin1 could mediate the degradation of TVBMV NIb. Western blotting result showed that MYC:NIb accumulation in the \textit{N. benthamiana} leaves overexpressing NbBeclin1:eGFP was reduced compared to that in the control plants (Figure 6D), indicating that overexpression of NbBeclin1 could enhance the degradation of TVBMV NIb. The RT-qPCR results showed that, by 2 dpai, the accumulation level of TVBMV RNA in the NbBeclin1-overexpressing leaves was significantly lower than that in the control (Figure 6E), indicating that NbBeclin1 could reduce TVBMV replication.

These results indicate that TVBMV infection can activate the autophagy pathway, and NbBeclin1 can interact with NIb and mediate its degradation.
The role of NbRPL1 in NbBeclin1-mediated in vivo degradation of NIb was determined through co-expressing NbBeclin1:eGFP, MYC:NIb, and HA:NbRPL1 in *N. benthamiana* leaves. Western blotting result showed that MYC:NIb accumulation in the HA:NbRPL1-overexpressing leaves was higher than that in the control plant leaves (Figure 6F), indicating that NbRPL1 overexpression reduced the NbBeclin1-mediated degradation of NIb. The RT-qPCR results showed that, by 2 dpai, the accumulation level of TVBMV RNA in the HA:NbRPL1- and NbBeclin1-overexpressing *N. benthamiana* leaves was significantly higher than that in the NbBeclin1-overexpressing *N. benthamiana* leaves (Figure 6G). These results indicated that NbRPL1 could promote TVBMV replication through reducing the degradation of NIb by NbBeclin1.

Considering that both NbRPL1 and NbBeclin1 can bind TVBMV NIb, it is reasonable to hypothesize that NbRPL1 interferes the NbBeclin1-mediated NIb degradation through competing with NbBeclin1 for NIb. We conducted a pull-down assay to determine the effect of NbRPL1 on NbBeclin1 and NIb interaction. Mixtures with HIS:MBP:MYC:NIb, HIS:eGFP:NbBeclin1 and different concentrations of HIS:HA:NbRPL1 were incubated with GFP-Trap_A beads followed by western blotting analyses using MYC-, HA-, and GFP-specific antibodies. The results showed that increase of HIS:HA:NbRPL1 concentration reduced the amount of HIS:MBP:MYC:NIb bound by HIS:eGFP:NbBeclin1 (Figure 6H). These results indicate that NbRPL1 can compete with NbBeclin1 for TVBMV NIb *in vitro*.

**DISCUSSION**

In this study, we determined that chloroplast ribosomal protein NbRPL1 interacts with TVBMV NIb in the 6K2-associated VRCs in chloroplasts. The cTP transit peptide domain of NbRPL1 is essential for the NbRPL1 chloroplast targeting. We also observed that NbRPL1 could reduce the degradation of NIb through interfering with the NbBeclin1-mediated autophagy pathway to promote TVBMV infection. This research advances our understanding
of the roles of chloroplast ribosomal proteins in potyviral pathogenesis, and provides a target for
engineering resistance to potyviruses.

Chloroplasts are important host organelles where many interactions between host factors
and pathogens occur. Several chloroplast localized viral proteins have been shown to play
critical roles in viral infection (Zhao et al., 2016; Bhattacharyya and Chakraborty, 2018).

Potyviruses form VRCs and replicate their genomes in chloroplasts. Potyvirus 6K2-induced
VRCs are transported from endoplasmic reticulum (ER) to chloroplasts via vesicular transport
and actomyosin motility systems (Wei et al., 2010). The ER-localized SNARE (soluble
N-ethylmaleimide-sensitive-factor attachment protein receptors) protein Syp71 has been shown
to co-localize with *Turnip mosaic virus* (*TuMV*) 6K2 in chloroplasts to mediate the fusion of
viral VRCs with chloroplasts. Silencing of *Syp71* expression arrested the formation of
chloroplast-bound 6K2 complexes, and inhibited viral infection (Wei et al., 2013). Our previous
study also showed that TVBMV 6K2 recruited chloroplast NbPsbO1, the Photosystem II
oxygen evolution complex protein of *Nicotiana benthamiana*, for virus replication, and
knockdown of *NbPsbO1* expression inhibited TVBMV and *Potato virus Y* replication in plants
(Geng et al., 2017). In this study, we found that NbRPL1, a 50S ribosomal protein L1,
accumulates in chloroplasts and interacts with TVBMV N Ib (Figure 1). During TVBMV
infection in the plant, viral N Ib hijacks NbRPL1 into the VRCs to ensure viral replication
(Figure 3). Overexpression of NbRPL1 promoted viral replication and cell-to-cell movement,
but not translation, while silencing of *NbRPL1* expression in *N. benthamiana* inhibited
TVBMV infection (Figures 4, 5). These results indicate that NbRPL1 is a host factor involved
in TVBMV infection.

Increasing evidence have shown that ribosomal proteins play active roles in potyvirus
infections. For example, TuMV requires both ribosomal protein S6 (RPS6) and ribosomal
protein S6 kinase for its infection in *N. benthamiana* (Rajamäki et al., 2017). PVA protein
translation has been shown to be promoted by *Arabidopsis thaliana* 60S acidic ribosomal
protein (Hafrén et al., 2013). Further, silencing ribosomal protein genes *RPL19*, *RPL13*, *RPL7*
or *RPS2* expression inhibited TuMV infection in *N. benthamiana* plants (Yang et al., 2009).
Tobacco etch virus protein translation is increased by 80S cytoplasmic ribosomes and 60S ribosomal subunits (Martínez and Daròs, 2014). Most of the reported ribosomal proteins that regulate viral infection are localized in the cytoplasm (Hafrán et al., 2013). Here, we found that the chloroplast ribosomal protein NbRPL1 can interact with TVBMV NIb and enhance TVBMV RNA and NIb accumulation in the 6K2-induced vesicles, but overexpression of NbRPL1 had little effect on TVBMV protein translation (Figures 1, 3–5).

Autophagy plays an antiviral role in plant–virus interactions (Yang et al., 2020). On the other hand, plant viruses can hijack the autophagy pathway to promote their pathogenesis (Yang et al., 2020). A previous study has shown that TuMV NIb interacts with the autophagy receptor NbBeclin1 and is degraded through autophagy-related pathways (Li et al., 2018). In this study, we also determined that NbBeclin1 can interact with TVBMV NIb and induce degradation of TVBMV NIb in a NbRPL1 dose-dependent manner (Figure 6), indicating that NbRPL1 is employed by TVBMV to antagonize autophagy and promote pathogenesis.

NbBeclin1 co-localizes with TVBMV VRCs in chloroplasts (Li et al., 2018). Our analyses also revealed that 9.39% and 4.85% of NbRPL1/NIb complexes co-localized with NbBeclin1 in cytoplasm and chloroplasts, respectively (Supplemental Figure S4). Considering the results described above, we postulate that the competition between NbRPL1-NIb and NbBeclin1-NIb interactions occurs in both cytoplasm and chloroplasts.

A recent study has shown that Phytophthora infestans effector AVRvnt1 interacts with chloroplast protein glycerate 3-kinase (GLYK) and activates Rpi-vnt1.1-mediated host resistance. A mutant GLYK lacking its cTP domain cannot activate Rpi-vnt1.1-mediated host resistance (Gao et al., 2020). Several plant viral proteins also contain cTPs that can autonomously target chloroplasts. For example, the cTP in the N-terminus of Cucumber necrosis virus CP is essential for chloroplast targeting (Hui et al., 2010). The Lolium latent virus CP also contains a typical cTP domain and localizes to chloroplasts (Vaira et al., 2018). The cTP domain of Tomato yellow leaf curl virus C4 protein plays an important role in translocating C4 protein from plasma membrane to chloroplasts upon activation of host defense mechanisms (Medina-Puche et al., 2020). Plant viruses lacking cTP need to hijack...
chloroplast factors with cTPs for targeting chloroplasts. Chloroplast protein plastocyanin contains a cTP and has been shown to interact with Potato virus X CP. Silencing of plastocyanin expression reduces the accumulation of PVX CP in chloroplasts (Qiao et al., 2009). chl-PGK has also been shown to be involved in targeting BaMV RNA to chloroplasts in N. benthamiana leaves (Cheng et al., 2013). NbcpHsp70-2 interacts with BaMV replicase, and its mislocalization affects BaMV replication (Huang et al., 2017). In our study, we found that the N1b-interacting chloroplast ribosomal protein NbRPL1 contains a cTP domain that is necessary for chloroplast targeting (Figure 2). We also found that during TVBMV infection, the NbRPL1/N1b complex co-localizes with TVBMV VRCs on chloroplasts (Figures 1, 3). Deletion of cTP from NbRPL1 decreased the chloroplast targeting of the NbRPL1/N1b complex (Figure 2). However, cTP is dispensable for the interaction between NbRPL1 and N1b (Figure 2F).

Based on the above results, we propose a working model for the role of NbRPL1 in TVBMV infection. Upon TVBMV infection, replicase N1b is recognized and then degraded by the NbBeclin1-mediated autophagy. To prevent this degradation, TVBMV recruits NbRPL1, which competes with NbBeclin1 for N1b, to prevent N1b degradation and promote TVBMV infection. NbRPL1 localizes in the VRCs in chloroplasts with the assistance of its cTP. The findings presented here advance our understanding of the roles of chloroplast ribosomal proteins in potyvirus pathogenesis and reveal an antiviral breeding strategy for potyvirus resistance.

**MATERIALS AND METHODS**

**Plant growth**

*Nicotiana benthamiana* seedlings were grown in pots containing isotonic soil matrix (pH 5.8–6.5) inside a growth chamber maintained at 22 ± 2 °C, with a 16/8 h (light/dark) photoperiod, and 70% relative humidity.
**Plasmid construction**

Total RNA was isolated from leaf samples harvested from *N. benthamiana* plants using TRIzol reagent. The RNA samples were treated with DNase I, and then used for cDNA synthesis as described previously (Cheng et al., 2020). The full-length chloroplast 50S ribosomal protein 1 gene (*NbRPL1*; Niben101Scf03253g02006.1) and Elongation factor thermo unstable gene (*NbEF-Tu*; Niben101Scf03816g02010) were PCR-amplified from *N. benthamiana* cDNA using a High-fidelity Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and cloned individually into the pEHISeGFP vector to produce pEHIS:eGFP:NbRPL1 and pEHIS:eGFP:NbEF-Tu using a Ligation-Free Cloning System (Applied Biological Materials, Richmond, BC, Canada) following the manufacturer’s instructions. The TVBMV N1b-coding sequence (JQ407082) was PCR-amplified from the cDNA from a TVBMV-infected plant, and cloned into pUTRMYC (an expression vector containing a 35S promoter, the 5′-untranslated region of TVBMV, and 2× MYC-coding sequence), pEHISMBPMYC (a vector containing a 35S promoter, and the Histone-, MBP-, and MYC-coding sequences), pCamYN (a vector containing the 5′ half of the YFP gene), pGBK7 (binding domain), and pGADT7 (activation domain). The *NbRPL1* sequence was also cloned into pCamYC (a vector containing the 3′ half of the YFP gene) and pUTRHA (an expression vector containing a 35S promoter, the 5′-untranslated region of TVBMV, and the HA-coding sequence). Expression vectors pUTRMYC, pUTRHA, pEHISMBPMYC, and pEHISMBP were constructed previously in our laboratory. Full-length *N. benthamiana Beclin1* (*NbBeclin1*; AY701316.1) was PCR-amplified, and cloned into the pCamYC, pGADT7, and pGBK7 vectors. In addition, the full-length *NbRPL1* and its deletion mutants (i.e., *NbRPL1*ΔcTP(N61aa), *NbRPL1*ΔN41aa, *NbRPL1*ΔN20aa, *NbRPL1*ΔN42-61aa::DsRed, *NbRPL1*ΔN21-61aa::DsRed, *NbRPL1*ΔN21-41aa::DsRed, *NbRPL1*Δ1-280aa, and *NbRPL1*281-340aa) were inserted individually into the XbaI/BamHI site in the pCameGFP and pCamDsRed vectors. For VIGS assays, a fragment representing a partial sequence of the *NbRPL1* gene (nucleotides 553–1002) was PCR-amplified, and inserted individually into a *Tobacco rattle virus* (TRV)-based VIGS vector (Liu et al., 2002) to produce pTRV2-NbRPL1. *RLUC* was inserted
between the Nlb and CP gene in the pCB301TVBMV^{AGDD} vector (a vector expressing a
replication-deficient mutant of TVBMV, Supplemental Figure S2) to produce
pCB301TVBMV^{AGDD}-RLUC. Further, a 35S-FLUC-NOS sequence was cloned into the
pCB301TVBMV^{AGDD}-RLUC vector to produce pCB301TVBMV^{AGDD}-RLUC//FLUC
(Supplementary Figure S2). Primers used in this study are listed in Table S1.

**Virus inoculation and transient gene expression**

Plasmids pCamTVBMV-GFP, pCamTVBMV^{ΔP3NPIPO}-GFP, pTRV1, pTRV2,
pCB301TVBMV^{AGDD}-RLUC//FLUC, and various transient expression vectors were
individually transformed into *Agrobacterium tumefaciens* strain GV3101. The transformed *A.
tumefaciens* cell cultures were maintained as described previously (Cheng et al., 2020). After incubation in an induction buffer containing 10 mM MES (pH 5.6), 10 mM MgCl₂, and 200 μM acetosyringone, the cell cultures were pelleted individually, and then diluted to OD₆₀₀ = 0.5 for virus inoculations, or to OD₆₀₀ = 0.2 for transient expression and BiFC assays in four-week-old *N. benthamiana* plants through agroinfiltration using 1 mL needleless syringes.

**In vitro pull-down and in vivo Co-IP assays**

*Escherichia coli* cells (Rossetta, DE3) transformed with pEHIS:eGFP, pEHIS:eGFP:NbRPL1,
pEHIS:eGFP:NbEF-Tu, or pEHIS:MBP:MYC:Nlb were grown on an orbital shaker (220 rpm) at 28 °C until an OD₆₀₀ of 0.5 was attained. After the addition of IPTG (0.4 mM for the pEHIS:eGFP-transformed cells or 2 mM for the pEHIS:MBP-transformed cells), the cultures were incubated again on the shaker for 12 h at 16 °C. The resulting cultures were pelleted by centrifugation at 8000 rpm, resuspended separately in 20 mL lysis buffer (0.3 M NaCl, 10 mM imidazole, 1 mM DTT, 0.25 mM PMSF, and 0.5 mg/mL lysozyme in PBS [pH 7.4]), and incubated for 30 min at 37 °C. The cells were disrupted by sonication on ice, and pelleted by centrifugation for 10 min at 8000 rpm at 4 °C. The supernatant of each sample was collected into a new centrifuge tube, and centrifuged for 20 min at 8000 rpm at 4 °C. The resulting supernatant was collected again after filtering through a 0.22 μm filter. Recombinant proteins in each filtered supernatant were purified using a high-affinity Ni-charged resin (GenScript,
New Jersey, USA), and the captured proteins were dialyzed overnight in a dialysis buffer (0.3 M NaCl, 1 M Tris-HCl [pH 7.5], and 1 mM DTT in PBS [pH 7.4]) at 4 °C. Twenty micrograms of purified HIS:eGFP:NbRPL1 or HIS:GFP:NbEF-Tu was added to a 1.5 mL centrifuge tube followed by the addition of 20 μg HIS:MBP:MYC:Nltb, 500 μL dilution buffer (pH 7.4), and 20 μL GFP-Trap_A beads (Chromotek, Munich, Germany). The centrifuge tubes were incubated for 2 h on ice on an orbital shaker. The beads were rinsed ten times with PBS (pH 7.4), and then resuspended in 1× SDS-sample buffer. After 10 min boiling and then 10 min incubation on ice, the proteins were separated by SDS-PAGE, and subsequently transferred onto nitrocellulose membranes. The membranes were probed with an MYC-specific antibody (Abways Technology, Shanghai, China). In vivo Co-IP assays were performed using a method reported previously (Geng et al., 2017). Western blotting assay was conducted as described previously (Cheng et al., 2020).

Y2H assay

The Y2H assay was performed according to the manufacturer’s instructions (Clontech, Mountain View, CA, USA). Briefly, yeast cell strain Y2H Gold was transformed with various expression constructs and then grown on a selection medium (SD/-Trp/-Leu) for 3 days. The selected colonies were then grown on a highly stringent selection medium supplemented with X-α-Gal (e.g., SD/-Trp/-Leu/-His/-Ade/+ X-α-Gal) for 4 days.

Subcellular localization and BiFC assays

Subcellular localization and BiFC assays were performed using N. benthamiana leaves. Fluorescence from GFP, YFP or DsRed in leaf cells was examined and imaged at 36 hpi under a Zeiss LSM800 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany). The excitation wavelength for GFP was set at 488 nm (argon ion laser) and the emission was captured at 505–545 nm. The excitation wavelength for YFP was set at 514 nm (argon ion laser) and the emission was captured at 560–585 nm. The excitation wavelength for DsRed was set at 543 nm and the emission was captured at 590–620 nm. Autofluorescence from chloroplasts was captured at 675–720 nm. Confocal images were created using a pinhole.
size of 1 AU with gains of 680, maximum light intensity value for the samples expressing YFP and eGFP, and 20% of maximum light intensity value for the samples expressing DsRed, mCherry, and autofluorescence from chloroplasts. The captured images were further processed using the Zeiss LSM Image Examiner version 4.0 or ZEN blue version 2.1 software (Leica Microsystems, Wetzlar, Germany).

VIGS

Plasmids pTRV2-NbRPL1, pTRV2-GUS, and pTRV1 were individually transformed into *A. tumefaciens* strain GV3101 cells. After propagation and incubation, each *A. tumefaciens* culture was pelleted and diluted to OD\(_{600}\) of 0.5. The *A. tumefaciens* culture harboring pTRV1 was mixed with the *A. tumefaciens* culture harboring pTRV2-NbRPL1 (the virus is referred to as TRV-NbRPL1) or pTRV2-GUS (referred to as TRV-GUS) at a ratio of 1:1 (v/v). The mixed cultures were separately infiltrated into the leaves of *N. benthamiana* plants. At 7 dpai, the leaves above the infiltrated leaves of the assayed plants were further infiltrated with an *A. tumefaciens* culture (OD\(_{600}\) = 0.5) carrying pCamTVBMV-GFP or pCamTVBMV\(^{\Delta P3NPO}\)-GFP.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Primers used in RT-qPCR were designed using an online tool (http://www.oligoarchitect.com/SYBRGreenSearchServlet), and are listed in Table S1. Total RNA was extracted from leaf samples using TRIzol reagent. Reverse transcription (10 μL each) was conducted using 2 μL of 5× HiScript II qRT Super Mix II reagent (Vazyme, Nanjing, China) and 500 ng total RNA. Quantitative PCR reactions (20 μL each) were conducted using 10 μL 2× ChamQ SYBR qPCR Master Mix reagent (Vazyme, Nanjing, China), 10 μM forward primer, 10 μM reverse primer, and 1 μL diluted cDNA on a LightCycler\(^\circledR\) 96 machine (Roche, Basel, Switzerland). Relative gene expression was calculated using the 2\(^{-\Delta\Delta Ct}\) method as described previously (Livak and Schmittgen, 2001). *NbEF1α* expression was used as an internal control. All experiments were repeated three times.
Protoplast preparations

Protoplast isolation from 4-week-old *N. benthamiana* plant leaves and their transfections were performed as described previously (Schweiger and Schwenkert, 2014). After transfection, protoplasts were incubated at 26 °C for 12 h and extracted for total RNA prior to RT-qPCR.

Dual-luciferase activity assay

*A. tumefaciens* cells carrying pCB301TVBMV$^{AGDD}$-RLUC//FLUC (Supplementary Figure S3) were cultured, pelleted, induced, and then diluted to $OD_{600} = 0.05$. The diluted cell suspension was infiltrated into leaves of the NbRPL1-overexpressing or control *N. benthamiana* plants to determine the effect of NbRPL1-silencing on virus accumulation. At 60 hpa, leaf discs (1 cm in diameter each) were collected from the infiltrated leaves and placed inside 2.0 mL centrifuge tubes. Each centrifuge tube was added with 150 μL lysis buffer (Vazyme, Nanjing, China), vortexed for 15 min, and then centrifuged for 2 min at 12000 rpm. The resulting supernatant was collected from each tube and was used for luciferase activity assays using the Dual-Luciferase Reporter Assay Kit (Vazyme, Nanjing, China). The experiment was repeated three times.

Statistical Analyses

The data shown in this study are means of three independent experiments. When two sets of data are compared, they were analyzed by the Student’s *t*-test. The *P* values less than 0.05 are indicated as *, and the *P* values less than 0.01 are indicated as **. ImageJ software was used to quantify the intensity of the bands in Western blotting.

Accession Numbers

The sequences of *NbRPL1* (accession number: Niben101Scf03253g02006.1), *NbEF-Tu* (accession number: Niben101Scf03816g02010.1), and *NbBeclin1* (accession number: AY701316.1) can be found in the SOL Genomics Network (SGN, https://solgenomics.net/) and NCBI.

Supplemental Data
Supplemental Figure S1. Peptides of NbRPL1 and NbEF-Tu identified through liquid chromatography tandem mass spectrometry.

Supplemental Figure S2. A schematic diagram of plasmid pCB301TVBMV\textsuperscript{AGDD}-RLUC//FLUC.

Supplemental Figure S3. Relative expression of autophagy-associated genes.

Supplemental Figure S4. Co-localization of the Nlb/NbRPL1 complex with NbBeclin1.

Supplemental Table S1. Primers used in this study.

Figure legends

**Figure 1. NbRPL1 physically interacts with TVBMV Nlb in vitro and in vivo.** A, Determination of interactions between HIS:eGFP:NbRPL1, HIS:eGFP:NbEF-Tu, and HIS:MBP:MYC:Nlb using in vitro pull-down assay. The combination of HIS:eGFP and HIS:MBP:MYC:Nlb was used as the negative control. Pull-down assays were performed using GFP-Trap_A beads followed by Western blotting analyses using an MYC-specific or a GFP-specific antibody. B, BiFC analysis of the interaction between Nlb:YN and NbRPL1:YC in *N. benthamiana* leaf cells. Co-expression of Nlb:YN and YC or YN and NbRPL1:YC were used as negative controls. C, Confocal micrographs of *N. benthamiana* leaves agroinfiltrated with plasmids expressing NbRPL1:eGFP or NbRPL1:DsRed. Images of *N. benthamiana* leaf cells were taken at 36 h post-agroinfiltration (hpai). Scale bars = 10 μm.

**Figure 2. Identification of the chloroplast transit peptide (cTP) domain, and its effects on the subcellular distribution of NbRPL1 and the interaction between Nlb and NbRPL1.** A, Prediction of cTP in NbRPL1. B, SDS-PAGE analysis of NbRPL1:DsRed expressed in the non-infected and TVBMV-infected *N. benthamiana* leaves. Sizes of the bands of the molecular weight marker are shown on the left. C, Schematic representations of NbRPL1 and its deletion mutants. D and E, Confocal micrographs of *N. benthamiana* leaf cells expressing NbRPL1\textsuperscript{ΔcTP(N61aa)}:DsRed, NbRPL1\textsuperscript{ΔN41aa}:DsRed, NbRPL1\textsuperscript{ΔN20aa}:DsRed, NbRPL1\textsuperscript{ΔN42-61aa}:DsRed, NbRPL1\textsuperscript{ΔN21-61aa}:DsRed, and NbRPL1\textsuperscript{ΔN21-42aa}:DsRed (D), and NbRPL1\textsuperscript{1-280aa}:DsRed and NbRPL1\textsuperscript{281-340aa}:DsRed (E). F, BiFC analysis of the interaction...
between NIb:YN and NbRPL1ΔcTP:YC. Co-expression of YN and NbRPL1ΔcTP:YC was used as a negative control. Images of *N. benthamiana* leaf cells were captured under a confocal microscope at 36 hpai. Scale bars = 10 μm.

**Figure 3.** The NbRPL1/NIb complex co-localizes with TVBMV 6K2 on chloroplasts upon TVBMV infection. A, Co-localization of NIb:YN, NbRPL1:YC, and 6K2:DsRed in *N. benthamiana* leaf cells. Co-expressions of NIb:YN, YC, and 6K2:DsRed or YN, NbRPL1:YC, and 6K2:DsRed were used as negative controls. White arrowheads indicate the co-localization of the NIb:YN/NbRPL1:YC complex with 6K2:DsRed. B, Subcellular distributions of NIb:YN, NbRPL1:YC, and H2B:mCherry or NIb:YN, NbRPL1:YC, and AtPDLP1:DsRed in *N. benthamiana* leaf cells. H2B:mCherry is a nuclear marker and AtPDLP1:DsRed is a plasmodesmata marker. C, Co-localization of NIb:YN, NbRPL1:YC, and 6K2:DsRed in the TVBMV-infected *N. benthamiana* leaf cells. White arrowheads indicate the co-localization of the NIb:YN/NbRPL1:YC complex with 6K2:DsRed. Images were taken under a confocal microscope at 36 hpai. Scale bars = 10 μm.

**Figure 4.** Silencing of *NbRPL1* expression affects TVBMV infection and the subcellular distribution of NIb in *N. benthamiana* plants. A, Phenotypes of the *NbRPL1*-silenced (TRV-NbRPL1) and non-silenced (TRV-GUS) *N. benthamiana* plants at 8 dpai. B, Relative expression of *NbRPL1* in the systemic leaves of the *NbRPL1*-silenced and non-silenced *N. benthamiana* plants was determined through RT-qPCR. Error bars indicate the standard deviations of three biological replicates per treatment. Statistical significance was determined using a two-tailed Student’s *t*-test (**P < 0.01). C, Phenotypes of the *NbRPL1*-silenced and non-silenced *N. benthamiana* plants at 6 days post TVBMV-GFP inoculation (dpi). Plants were photographed under UV illumination. D, Western blotting analysis of TVBMV CP accumulation in the systemic leaves of the TVBMV-GFP-inoculated *NbRPL1*-silenced or non-silenced *N. benthamiana* plants at 6 days post TVBMV-GFP inoculation. The Coomassie Brilliant Blue R-250 (CBB)-stained gel shows sample loadings. E, RT-PCR detection of
TVBMV RNA accumulation in the systemic leaves of the TVBMV-GFP-inoculated

*NbRPL1*-silenced or non-silenced *N. benthamiana* plants at 6 dpi using TVBMV CP gene

specific primers. F, GFP fluorescence in the *NbRPL1*-silenced *N. benthamiana* leaves. Images

of TVBMV-GFP-inoculated non-silenced or *NbRPL1*-silenced leaves were taken under

normal light or UV illumination at 72 hpa. G, Western blotting analysis of TVBMV CP

accumulation in the TVBMV-GFP-inoculated *NbRPL1*-silenced or non-silenced *N.

*benthamiana* leaves at 72 hpa. The Coomassie Brilliant Blue R-250 (CBB)-stained gel shows

sample loadings. H, RT-PCR detection of TVBMV RNA accumulation in the

TVBMV-GFP-inoculated *NbRPL1*-silenced or non-silenced *N. benthamiana* leaves at 72 hpa

using the TVBMV CP gene specific primers. I, RT-qPCR analysis of TVBMV-ΔP3NPIPO-GFP

RNA accumulation in the TVBMV-GFP-inoculated *NbRPL1*-silenced or non-silenced *N.

*benthamiana* leaves at 36 hpa using the TVBMV CP gene specific primers. Error bars

indicate the standard deviation of three biological replicates per treatment. Statistical

significance was determined using a two-tailed Student’s *t*-test (**P < 0.01). J, Localization of

6K2:DsRed in the *NbRPL1*-silenced or non-silenced *N. benthamiana* leaves. K,

Co-localization of Nlb:mCherry and TVBMV-6K2:GFP in the *NbRPL1*-silenced or

non-silenced *N. benthamiana* leaves. White arrowheads indicate the co-localization of

6K2:GFP and Nlb:mCherry. Scale bars = 10 μm.

**Figure 5. Overexpression of NbRPL1 in N. benthamiana leaves increases TVBMV**

cell-to-cell movement and RNA accumulation, and increases the accumulation of

TVBMV Nlb. A, Micrographs of TVBMV-GFP cell-to-cell movement in the control and

*NbRPL1*-overexpressing *N. benthamiana* leaves. Images were taken at 96 hpa. Green

fluorescence represents the GFP-tagged TVBMV. Scale bars = 500 μm. B, The mean number

of TVBMV-GFP-infected cells in each examined field. The number of TVBMV-GFP-infected

cells in each locus was counted under the stereo fluorescence microscope. For each treatment,

30 infection loci were counted. In the NbRPL1-overexpressing experiment, the boxplots

represent the data dispersion around the median with the position of the lower limit value (18),
the lower quartile (28.5), the median (45.5), the upper quartile (58.75) and the upper limit (91). In the control, the boxplots represent the data dispersion around the median with the position of the lower limit value (10), the lower quartile (23), the median (28), the upper quartile (33) and the upper limit value (46). Statistical significance was calculated using a two-tailed Student’s $t$-test (**$P < 0.01$). 

C. Effect of NbRPL1 overexpression on viral protein translation efficiency in *N. benthamiana* leaves. Relative activities of RLUC and FLUC in the pCB301TVBMV$_{AGDD}$-RLUC//FLUC-inoculated leaves were determined at 60 hpa. Error bars indicate the standard deviation of three biological replicates per treatment. D, RT-qPCR detection of TVBMV genomic RNA in the NbRPL1-overexpressing and control *N. benthamiana* protoplasts using TVBMV CP gene specific primers. Expression of *NbEF1a* was used as an internal control. Error bars indicate the standard deviation of three biological replicates per treatment. Statistical significance was determined using a two-tailed Student’s $t$-test (*$P < 0.05$). E, RT-qPCR detection of TVBMV$_{AP3NPIPO}$-GFP RNA in the NbRPL1-overexpressing or control *N. benthamiana* leaves using TVBMV CP gene specific primers. Expression of *NbEF1a* was used as an internal control. Error bars indicate the standard deviation of three biological replicates per treatment. Statistical significance was calculated using a two-tailed Student’s $t$-test (*$P < 0.05$). F, Western blotting detection of MYC:Nlb co-expressed with NbRPL1:DsRed or DsRed in the TVBMV-infected *N. benthamiana* leaves at 2 dpai using MYC-specific and mCherry-specific antibodies, respectively. Band intensities were measured using the ImageJ software. The Coomassie Brilliant Blue R-250 (CBB)-stained gel shows sample loadings.

Figure 6. NbRPL1 competes with NbBeclin1 for Nlb. A, Co-immunoprecipitation (Co-IP) analysis of NbBeclin1:eGFP and MYC:Nlb *in vivo* interaction. Combination of eGFP and MYC:Nlb was used as the negative control. The Co-IP analysis was performed with GFP-Trap_A beads followed by western blotting detection using an MYC-specific antibody. B, Determination of Nlb and NbBeclin1 interaction through Y2H assay. Yeast cells were co-transformed with BD-Nlb and AD-NbBeclin1 or AD-Nlb and BD-NbBeclin1. The
transformed cells were grown on the SD/-Trp/-Leu/-His/-Ade selection medium supplemented with X-α-Gal for 4 days. Y2H cells co-transformed with AD-T-ant and BD-p53 were used as the positive control, whereas Y2H cells co-transformed with AD-T-ant and BD-lam, BD-NIb and AD, AD-NIb and BD, BD and AD-NbBeclin1 or AD and BD-NbBeclin1 were used as negative controls. BD, pGBK7; AD, pGADT7. C, BiFC analysis of NIb:YN and NbBeclin1:YC interaction in N. benthamiana leaves. NIb:YN and YC or YN and NbBeclin1:YC co-expressing N. benthamiana leaves were used as negative controls. Confocal images of leaf cells were taken at 36 hpa. Scale bars = 10 μm. D, Western blotting assay of total protein extracts from N. benthamiana leaves co-expressing NbBeclin1:eGFP and MYC:NIb using a GFP specific or a MYC specific antibody at 2 dpai. A N. benthamiana leaf sample co-expressing NbBeclin1:eGFP and MYC:NIb was used as the negative control. The Coomassie Brilliant Blue R-250 (CBB)-stained gel shows sample loadings. E, RT-qPCR detection of TVBMV RNA in the N. benthamiana leaves co-expressing TVBMV and NbBeclin1:eGFP at 2 dpai. Co-expression of TVBMV and eGFP in N. benthamiana leaves was used as the negative control. Expression of NbEF1α was used as an internal control. Error bars indicate the standard deviation of three biological replicates per treatment. Statistical significance was determined using a two-tailed Student’s t-test (***P < 0.01). F, Western blotting analysis of total protein extracts from N. benthamiana leaves co-expressing HA:NbRPL1, NbBeclin1:eGFP, and MYC:NIb using a HA specific, a GFP specific or a MYC-specific antibody at 2 dpai. Co-expression of NbBeclin1:eGFP, MYC:NIb, and the empty vector in N. benthamiana leaves was used as a negative control. The Coomassie Brilliant Blue R-250 (CBB)-stained gel shows sample loadings. G, RT-qPCR detection of TVBMV RNA in the N. benthamiana leaves co-expressing NbBeclin1:eGFP and HA:NbRPL1 at 2 dpai. Co-expression of NbBeclin1:eGFP and empty vector in N. benthamiana leaves was used as the negative control. Expression of NbEF1α was used as an internal control. Error bars indicate the standard deviation of three biological replicates per treatment. Statistical significance was determined using a two-tailed Student’s t-test (*P < 0.05). H, Competitive binding of NbRPL1 and NbBeclin1 to NIb in vitro. The mixed protein
samples of HIS:MBP:MYC:N1b, HIS:eGFP:NbBeclin1, and HIS:HA:NbRPL1 with different concentrations were adsorbed with GFP-Trap_A beads followed by western blotting detection using a HA specific, a GFP specific or a MYC specific antibody. Band intensities shown in (D), (F), and (H) were quantified with ImageJ software.

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### Table

| Protein | Length | Score | CS-score | cTP-length |
|---------|--------|-------|----------|------------|
| NbRPL1  | 340 aa | 0.499 | 10.182   | 61         |

**cTP sequence:**

MASTATSSLTSSLLSQELNPNSLLFKPK

ALLFTPHKSFSLHPILHYKERKSNPGIVVA

**cTP cleave-site:** VVA↓A

### Diagram

#### A

- Protein Length Score CS-score cTP-length
- NbRPL1 340 aa 0.499 10.182 61

**cTP sequence:**

MASTATSSLTSSLLSQELNPNSLLFKPK

ALLFTPHKSFSLHPILHYKERKSNPGIVVA

**cTP cleave-site:** VVA↓A

#### B

- (kDa)
  - Marker
  - NbRPL1:DsRed
  - NbRPL1:DsRed
  - NbRPL1:DsRed

| Marker | NbRPL1:DsRed | NbRPL1:DsRed | NbRPL1:DsRed |
|--------|--------------|--------------|--------------|
| 95     | 40.5%        | 29.5%        |              |
| 72     | 59.5%        | 70.5%        |              |
| 55     |              |              |              |
| 43     |              |              |              |

#### C

- NbRPL1
  - full length
  - ΔA281-340aa
  - ΔA1-280aa
  - ΔcTP(N61aa)
  - ΔN41aa
  - ΔN20aa
  - ΔN42-61aa
  - ΔN21-41aa
  - ΔN21-61aa

#### D

- DsRed
- Chloroplast
- Merged

#### E

- YFP
- Chloroplast
- Merged

#### F

- YN+ NbRPL1
- ΔcTP-YC

- YN+ NbRPL1
- ΔcTP-YC
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