Beclin1 restricts RNA virus infection in plants through suppression and degradation of the viral polymerase

Fangfang Li1,2, Changwei Zhang1,3, Yinzi Li1,2, Guanwei Wu1,2, Xilin Hou3, Xueping Zhou4 & Aiming Wang1,2

Autophagy emerges as an essential immunity defense against intracellular pathogens. Here we report that turnip mosaic virus (TuMV) infection activates autophagy in plants and that Beclin1 (ATG6), a core component of autophagy, inhibits virus replication. Beclin1 interacts with NiB, the RNA-dependent RNA polymerase (RdRp) of TuMV, via the highly conserved GDD motif and the interaction complex is targeted for autophagic degradation likely through the adaptor protein ATG8a. Beclin1-mediated NiB degradation is inhibited by autophagy inhibitors. Deficiency of Beclin1 or ATG8a enhances NiB accumulation and promotes viral infection and vice versa. These data suggest that Beclin1 may be a selective autophagy receptor. Overexpression of a Beclin1 truncation mutant that binds to NiB but lacks the ability to mediate NiB degradation also inhibits virus replication. The Beclin1-RdRp interaction further extends to several RNA viruses. Thus Beclin1 restricts viral infection through suppression and also likely autophagic degradation of the viral RdRp.
Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved intracellular process in which double membrane-bound sack-like vesicles, termed autophagosomes, are biosynthesized to enclose and deliver cytoplasmic materials including macromolecular complexes and organelles to the lysosome/vacuole for degradation/recycling. Autophagy may be divided into several major steps, including induction, nucleation and expansion of the phagophore, maturation of the autophagosomes, and docking/fusion with the vacuole or lysosome, followed by degradation and breakdown. Extensive genetic screens of yeast mutants have led to the identification of ~40 autophagy-related genes (ATGs), and approximately half of them are indispensable for the formation of autophagosomes. Most of these key ATGs in yeast have orthologs in mammals and plants. During canonical autophagy, the corresponding gene products of the key ATGs form several functionally distinct groups to assemble the core autophagy machinery. One of them is the phosphatidylinositol 3-kinase (PI3K) complex containing PI3K/VPS34, VPS15/P150, and Beclin1/ATG6/VPS30. Initially, autophagy was assumed to be a rather unspecific, adaptive bulk catabolic process to provide energy and recycle nutrients for survival upon starvation stress. It is now well established that autophagy can operate either in a non-selective or a highly selective mode, depending on the type of cargo to be degraded. Selective autophagy can target a variety of substrates ranging from individual proteins and protein complexes to entire organelles and invading microbes. It involves the recruitment of specific receptor and adaptor proteins that simultaneously interact with specific cargoes and autophagy modifiers. Autophagy is activated during infection by diverse viruses in metazoa. Although autophagy has been suggested to play both antiviral and proviral roles, compelling evidence suggests that autophagy participates in innate and adaptive immune responses to eliminate pathogenic viruses. For instance, in Drosophila, vesicular stomatitis virus infection induces autophagy, and depletion of ATGs by RNAi leads to increased virus replication and fly mortality. In mice, ATG5 plays an essential role against lethal infection of autophagosomes. Most of these key ATGs in yeast have orthologs in mammals and plants. During canonical autophagy, the corresponding gene products of the key ATGs form several functionally distinct groups to assemble the core autophagy machinery. One of them is the phosphatidylinositol 3-kinase (PI3K) complex containing PI3K/VPS34, VPS15/P150, and Beclin1/ATG6/VPS30. Initially, autophagy was assumed to be a rather unspecific, adaptive bulk catabolic process to provide energy and recycle nutrients for survival upon starvation stress. It is now well established that autophagy can operate either in a non-selective or a highly selective mode, depending on the type of cargo to be degraded. Selective autophagy can target a variety of substrates ranging from individual proteins and protein complexes to entire organelles and invading microbes. It involves the recruitment of specific receptor and adaptor proteins that simultaneously interact with specific cargoes and autophagy modifiers. Autophagy is activated during infection by diverse viruses in metazoa. Although autophagy has been suggested to play both antiviral and proviral roles, compelling evidence suggests that autophagy participates in innate and adaptive immune responses to eliminate pathogenic viruses. For instance, in Drosophila, vesicular stomatitis virus infection induces autophagy, and depletion of ATGs by RNAi leads to increased virus replication and fly mortality. In mice, ATG5 plays an essential role against lethal infection of autophagosomes. Most of these key ATGs in yeast have orthologs in mammals and plants.

Results
TuMV infection activates autophagy. The autophagy pathway genes are constitutively expressed at the basal level to maintain homeostasis and are upregulated in response to abiotic and biotic stress. To determine whether TuMV infection induces autophagy, we used yellow fluorescent protein (YFP)-tagged N. benthamiana ATG8a (YFP-NbATG8a) as a autophagosome marker to monitor autophagy. Compared to mock-infiltrated N. benthamiana plants, the number of the punctate YFP fluorescent structures representing pre-autophagosomal or autophagosomal structures in TuMV-infected plants increased by more than two-fold. These data indicate that TuMV infection activates autophagy.

We further examined the expression of ATG genes in these plants using quantitative real-time reverse-transcription PCR (qRT-PCR). The expression levels of all tested nine ATG genes, including NbBeclin1, NbPI3K, NbVPS15, NbATG2, NbATG9, NbATG3, NbATG5, NbATG7, and NbATG8a, were significantly upregulated in TuMV-infected leaves (Fig. 1c) or upper non-inoculated leaves (Fig. 1d), compared to their respective counterparts in mock- or TuMV-ΔGDD-inoculated plants. These data indicate that TuMV infection activates autophagy.

NbBeclin1 interacts with the viral RdRp. As NbBeclin1 (ATG6 in yeast or Arabidopsis) was the most upregulated gene
**Fig. 1** TuMV infection activates autophagy. 

**a** Confocal micrographs showing *N. benthamiana* leaf cells co-infiltrated with *Agrobacterium* harboring a YFP-NbATG8a expression construct and *Agrobacterium* carrying an empty vector (mock) or a TuMV infectious clone (TuMV) or a TuMV replication-defective mutant (TuMV-ΔGDD) at 60 h post infiltration (hpi). Bars, 50 μm. 

**b** The average number of YFP-NbATG8a spots per 10 cells. Infiltration experiments were repeated three times and 60 cells in total were counted for the punctate spots. The average number was calculated using 10 cells as a unit. Values represent the mean spots ± standard deviation (SD) per 10 cells. 

**c, d** Effects of viral infection on the expression of autophagy components at 3 and 7 days post infiltration (dpi). *N. benthamiana* leaves were agroinfiltrated with mock, TuMV, or TuMV-ΔGDD. Total RNAs were extracted from infiltrated zones at 3 dpi (**c**) or from newly emerged leaves at 7 dpi (**d**). Values represent the mean relative to the mock-treated plants (*n* = 3 biological replicates) and were normalized with NbActin as an internal reference. 

**e, f** Immunoblotting analysis of total protein isolated from upper non-inoculated leaves of plants agroinfiltrated with mock, TuMV, and TuMV-ΔGDD at 7 dpi with anti-Beclin1 (**e**) or anti-ATG8 (**f**) antibody. Coomassie Brilliant Blue R-250 (CBB)-stained Rubisco large subunit serves as a loading control. 

**g** Representative TEM images from upper non-inoculated leaves of *N. benthamiana* plants infected with mock, TuMV, and TuMV-ΔGDD at 7 dpi. Obvious autophagic structures (red arrows) were observed in TuMV-infected samples and the corresponding region in the white box in the left panel is magnified in the right panel. Cp chloroplast, CW cell wall, P particle, S starch, V vacuole. Bars = 1 μm. 

**h** The number of typical double-membrane autophagosomes in different samples in **g**. Experiments were repeated twice and typical autophagic structures were counted in 30 cells in each treatment. Values represent the mean number of autophagosomes ±SD per 10 cells. 

**b, c, d, h** Data were analyzed using Student’s t-test and asterisks denote significant differences between mock (or TuMV-ΔGDD) and TuMV-infected leaves (two-sided, *P* < 0.05, **P** < 0.01)
Fig. 2 NbBeclin1 interacts with NbIb. a Yeast-two hybrid (Y2H) assays for possible interactions between NbBeclin1 and each of the 11 TuMV proteins. NbBeclin1 and 11 viral proteins were fused with a GAL4 activation domain (AD-NbBeclin1) and a GAL4-binding domain (BD-P1, BD-HC-Pro, BD-P3, BD-P3N-PIPO, BD-6K1, BD-CI, BD-6K2, BD-Nla-VPg, BD-Nla-Pro, BD-Nb, BD-CP), respectively. Y2H Gold yeast cells co-transformed with the indicated plasmids were subjected to 10-fold serial dilutions and plated on synthetic dextrose (SD)/-Trp, -Leu, -His, -Ade or SD/-Trp, -Leu medium to screen for positive interactions at 3 days after transformation. Yeast co-transformed with AD-T7-T+BD-T7-53 serves as a positive control; yeast cells co-transformed with AD-NbBeclin1 and the empty BD or with the empty AD and BD-Nb are negative controls. b BiFC assays between NbBeclin1 and NbIb in the leaves of H2B-RFP transgenic N. benthamiana. Confocal imaging was performed at 48 hpi. NbBeclin1 and NbIb were fused to the N (YN) and C-terminal (YC) fragments of yellow fluorescent protein (YFP). The NbBeclin1-NbIb interaction led to the reconstituted fluorescence-competent structure and restoration of yellow fluorescence (green). Nuclei of tobacco leaf epidermal cells are indicated by the expression of H2B-RFP transgene (red). Bars, 50 μm. c Co-localization of NbIb-YFP with NbBeclin1-CFP in the leaf cells of H2B-RFP transgenic N. benthamiana by confocal microscopy at 48 hpi. Arrow indicates yellow fluorescence, which was produced from the overlapping of NbIb-YFP (green) and NbBeclin1-CFP (red). Bars, 50 μm. d Co-immunoprecipitation (Co-IP) analysis of NbBeclin1-CFP and Myc-NbIb in vivo. N. benthamiana leaves were co-infiltrated with A. tumefaciens cells harboring expression vectors to express NbBeclin1-CFP and Myc-NbIb (Lane 1), NbBeclin1-CFP and Myc-P3N-PIPO (Lane 2), Myc-NbIb and GFP (Lane 3), and GFP and Myc-P3N-PIPO (Lane 4). Leaf protein extracts were incubated with GFP-Trap®_MA magnetic agarose beads (ChromoTek). Samples before (Input) and after (IP) immunopurification were analyzed by immunoblotting using GFP or Myc antibody.
When Nb-YFP and NbBeclin1-CFP were co-expressed, some Nb-YFP was re-distributed to the NbBeclin1-CFP-labeled punctate structures in the cytoplasm (Fig. 2c). Co-immunoprecipitation (Co-IP) assay showed that green fluorescent protein (GFP) antibody could specifically co-purify NbBeclin1-CFP and Myc-NIb (Fig. 2d), confirming the presence of the NbBeclin1-NIb complex in planta.

AtATG6/VPS30-labeled punctate structures are co-distributed with ATG8, an established autophagosome marker. We thus tested the possible association of the Nb-NIb complex with autophagosomes. Co-expression and BiFC analyses showed that NbBeclin1-YFP and the Nb-NIb complex indeed co-localized with NbATG8a-CFP to the punctate structure within the cytoplasm (Fig. 3a–c). These results indicate that the NbBeclin1-NIb complex is physically associated with autophagosomes.

Since Nb is an essential component of the viral replication complex (VRC), we hypothesized that NbBeclin1 is associated with the VRC through its interaction with Nb. We thus conducted a co-localization assay using NbBeclin1-YFP and a modified TuMV infectious clone TuMV-CFP-Nlb (by insertion of cyan fluorescent protein (CFP) to the N-terminus of Nb in the infectious clone TuMV-6K2-mCherry). The 6K2-induced structure derived from viral expression is an established marker of the VRC. NbBeclin1-YFP co-localized with the 6K2-induced, CFP-Nlb-containing irregular structures in the cytoplasm in TuMV-infected leaf cells (Fig. 4a). A dsRNA reporter that recognizes dsRNA such as replicative RNA intermediates also bound to the NbBeclin1-interacting, 6K2-induced structures, further supporting that they were active VRCs (Fig. 4b). To test whether the NbBeclin1-bound VRC interacts with autophagosomes, NbATG8a-YFP or NbATG8a-CFP was transiently co-expressed with NbBeclin1-CFP or the pair of YN-NbBeclin1/YC-Nlb or YC-NbBeclin1/YN-Nlb in N. benthamiana infected by TuMV-6K2-mCherry. NbATG8a-YFP or NbATG8a-CFP was indeed associated with the 6K2-induced VRC, which co-localized with NbBeclin1 or the NbBeclin1–NIb interaction complex.
It was worthy to note that there was no co-localization between 6K2 and NbATG8a or NbBeclin1 when they were co-expressed together in the absence of viral replication in N. benthamiana leaves (Supplementary Fig. 4a–c). These results indicate that NbBeclin1 co-localizes with the VRC by binding to NbN.

**NbBeclin1 mediates NbN degradation likely via autophagy.** As NbBeclin1 may serve as a receptor protein for the autophagic clearance of viral proteins, we investigated whether NbBeclin1 has a role in the degradation of NbN. Immunoblotting analysis revealed that the levels of NbN-YFP markedly decreased when co-expressed with Myc-NbBeclin1 (Fig. 5a). A similar reduction was also observed when Myc-NbN was co-expressed with NbBeclin1-CFP (Fig. 5b). No obvious change in NbN or NbBeclin1 mRNA was observed when they were expressed alone or together (Supplementary Fig. 5a,b). To determine whether NbBeclin1-mediated NbN degradation holds true in the context of viral infection, we co-infiltrated N. benthamiana leaves with the infectious clone TuMV-CFP-NbN and an empty vector (Vec) or TuMV-CFP-NbN and Myc-NbBeclin1. Immunoblotting analysis confirmed that the levels of CFP-NbN from viral expression markedly decreased when co-expressed with Myc-NbBeclin1 (Fig. 5c). In contrast, overexpression of NbBeclin1 did not affect 6K2-GFP accumulation from non-viral expression (Fig. 5e). It is well known that virus intercellular movement does not occur for potyviruses until 72 hpi. We thus determined the viral RNA level in the primarily infected cells at 60 hpi by qRT-PCR. We found that overexpression of NbBeclin1 significantly inhibited viral RNA accumulation (Fig. 5d). These results suggest that NbBeclin1-mediated degradation of NbN and its complexes including VRCs inhibits viral replication.

Drug treatment with 3-methyladenine (3-MA) and E64d, two well-known autophagy inhibitors, was performed to test whether the autophagy pathway is responsible for NbBeclin1-mediated degradation of NbN. 3-MA treatment enhanced the accumulations
of NiB-YFP or CFP-NiB derived from viral expression (Supplementary Fig. 5c,d). In 3-MA-treated plants, the TuMV RNA level also increased (Supplementary Fig. 5e). E64d treatment showed similar results (Supplementary Fig. 5f,g). To observe the effect of impaired autophagy flux on the accumulation of autophagic bodies and viral genomic RNA, we conducted another drug treatment with the specific vacuolar ATPase inhibitor concanamycin A (Con A). Con A treatment led to the accumulation of significantly more autophagic bodies associated with VRCs and of enhanced levels of TuMV RNA at 72 hpi, compared to DMSO treatment, supporting the antiviral role of autophagy in viral infection (Supplementary Fig. 6).

Next, we determined whether NbBeclin1-mediated degradation of NiB was affected by autophagy inhibitors or by knockdown of NbATG8. Regardless of co-expression with NbBeclin1 or not, the levels of NiB-YFP or viral expression-derived CFP-NiB increased in 3-MA- or E64d-treated plants (Fig. 5f, g and Supplementary Fig. 5c,d,f,g). A TRV-based virus-induced gene silencing (VIGS) vector was employed to silence NbATG8a and NbATG8f, and knockdown of NbATG8a rather than NbATG8f remarkably inhibited NbBeclin1-mediated degradation of NiB-YFP or CFP-NiB (Fig. 5h, i and Supplementary Fig. 7a–c). A GFP protein that is relatively resistant to the autophagy pathway was used as a control (Fig. 5a, b and Supplementary Fig. 5h–k).
Mapping of the interacting domains. The protein domains required for the interaction between NbBeclin1 and NbIb were mapped by Y2H assays (Fig. 6a and Supplementary Fig. 8a–d). The C-terminal portion (containing the APG6 domain) of NbBeclin1 (NbBeclin1-C) and the RdRp domain of NbIb (NbIb-M) were identified to be responsible for the NbBeclin1 and NbIb interaction (Fig. 6a and Supplementary Fig. 8a–d). This observation was consistent with results from BiFC assays in planta (Supplementary Fig. 8e). The NbIb-interacting domain NbBeclin1-C was also essential for the punctate localization of NbBeclin1 (Supplementary Fig. 8f).

Since the conserved GDD motif is located in NbIb-M, we tested the importance of this motif with respect to the NbIb–NbBeclin1 interaction. Deletion of GDD abolished the interaction (Supplementary Fig. 8b,d,g). As NbIb-M is highly conserved among potyviruses, we investigated the potential interaction between NbBeclin1 and Nibs from other potyviruses, including PPV, SMV, and TEV. Positive interactions were detected by Y2H assays (Supplementary Fig. 8g); consistently, GDD deletion extirpated the interaction (Supplementary Fig. 8g).

For selective autophagy, receptor proteins require binding to adaptors to facilitate docking of autophagy substrates to the autophagosomes. To test whether NbBeclin1 binds to the autophagy adaptors ATG8 family proteins, the possible interaction between NbBeclin1 and NbATG8a or NbATG8f was analyzed. Y2H, BiFC, and Co-IP experiments consistently showed that NbBeclin1 interacted strongly with NbATG8a but not NbATG8f (Fig. 6b, c and Supplementary Fig. 9). The NbATG8-interacting domain was mapped to the N-terminal region of NbBeclin1 (NbBeclin1-N) (Fig. 6b, c and Supplementary Fig. 9). A web-based analysis (http://repeat.biol.ucy.ac.cy/iLIR) identified a potential ATG8 interacting motif (AIM) in NbBeclin1-N. The NbBeclin1-N AIM mutant (NbBeclin1-N^AIM) resulting from the replacement of EESSVFL with EESAVYA lost the ability to interact with NbATG8a (Fig. 6b, c and Supplementary Fig. 9). NbBeclin1^AIM was still able to form bright granules, interact with, and co-localize with NbIb in the cytoplasm (Supplementary Fig. 10a–c), consistent with the observation that the C-domain of NbBeclin1 (NbBeclin1-C) was required for NbIb interaction and its punctate localization (Fig. 6a and Supplementary Fig. 8e,f). Autophagic structures were analyzed by TEM in N. benthamiana leaves transiently expressing NbBeclin1, NbBeclin1^AIM, NbBeclin1-N, NbBeclin1-N^AIM, or NbBeclin1-C. Compared to buffer treatment (mock) and the expression of NbBeclin1^AIM, NbBeclin1-N^AIM, or NbBeclin1-C, the expression of NbBeclin1 or NbBeclin1-N induced the formation of the typical double-membrane autophagosomes (Fig. 6d–f). Further, an autophagic flux assay on NbBeclin1 and its truncated mutants was conducted. Treatment of YFP:NbATG8a-expressing N. benthamiana leaves with Con A increased the number of NbATG8a-labeled puncta in the presence of NbBeclin1 or NbBeclin1-N but not other NbBeclin1 mutants (including NbBeclin1^AIM, NbBeclin1-N^AIM, or NbBeclin1-C) (Supplementary Fig. 11a,b), indicating that NbBeclin1-N and its AIM was required to induce and enhance autophagy flux. These observations were also confirmed using western blot analyses (Supplementary Fig. 11c). The expression of NbBeclin1 or NbBeclin1-N, rather than NbBeclin1^AIM, NbBeclin1-N^AIM, or NbBeclin1-C, decreased the levels of YFP-NbATG8a protein (Supplementary Fig. 11c). Treatment of these samples with E64d, an inhibitor of vacuolar proteases, blocked NbBeclin1- or NbBeclin1-N-mediated YFP-NbATG8a degradation (Supplementary Fig. 11c). These data suggest that NbBeclin1 induces autophagy through interacting with the autophagy adaptor NbATG8a via AIM (located at the N-terminus) and serves as an autophagy receptor by interacting with cargo proteins via its C-terminus.

NbBeclin1 also suppresses NbIb independent of autophagy. NbBeclin1 co-localized and interacted with NbATG8a and NbIb to form puncta and induce autophagy flux in the cytoplasm (Fig. 7a, b), and mediated their degradation (Fig. 7b and Supplementary Fig. 11d). The AIM deletion mutant NbBeclin1^AIM lost this capacity (Fig. 7a, b and Supplementary Fig. 11d). Western blots showed that NbBeclin1-mediated degradation of NbATG8a and NbIb was obviously suppressed by the treatment of E64d or Con A (Supplementary Fig. 11d). These data suggest that NbBeclin1, as an autophagy cargo receptor to form autophagosomes and mediate cargo protein degradation, requires the AIM. Full-length NbBeclin1 (NbBeclin1-FL) and its truncated proteins (NbBeclin1-N and NbBeclin1-C) were compared for their ability to mediate NbIb degradation. Both NbBeclin1-N and NbBeclin1-C lost this ability (Fig. 7d and Supplementary Fig. 11d), suggesting that NbBeclin1-N (which binds to NbATG8a via AIM to trigger autophagy) and NbBeclin1-C (which interacts with NbIb) are both required for NbBeclin1-mediated NbIb degradation. Consistent with the requirement of GDD for the NbIb–NbBeclin1 interaction, NbBeclin1-FL failed to degrade NbIb^ΔGDD–YFP (Fig. 7d). The effect of NbBeclin1, NbBeclin1-N and NbBeclin1-C on TuMV replication was analyzed. It was surprising that NbBeclin1-C failed to degrade NbIb protein, but it was still able to suppress viral RNA replication (Fig. 7e). The expression of NbBeclin1 and NbBeclin1-C decreased viral RNA replication to approximately 8% and 25%, respectively (Fig. 7e). The fact that NbBeclin1-C is responsible for the NbBeclin1 and NbIb interaction (Fig. 6a and Supplementary Fig. 8a–e) prompted us to consider the possibility that the interaction between NbBeclin1 and NbIb may also interrupt viral replication independent of autophagy-mediated NbIb degradation. Serial deletions of NbBeclin1-C were created and

![Fig. 5 Overexpression of NbBeclin1 promotes autophagy-dependent degradation of NbIb and inhibits TuMV replication. **a, b** Immunoblotting of total protein extracts from the N. benthamiana leaves agroinfiltrated with buffer (−) or the plasmids indicated. The membrane was probed with GFP (@GFP), or Myc antibodies (@Myc). **c** Immunoblotting analysis of total protein extracts from leaves infiltrated with buffer (−) or TuMV-CFP-Nib together with an empty vector (Vector) or Myc-NbBeclin1 with antibodies against GFP or Myc. **d** Quantification of TuMV RNA levels by qRT-PCR. RNA was extracted from leaves agroinfiltrated with TuMV-CFP-Nib together with Vector or Myc-NbBeclin1 at 60 hpi. Values represent mean ± SD relative to plants infiltrated with TuMV-CFP-Nib and Vector (n = 3 biological replicates). The data were analyzed using Student’s t-test and asterisks denote significant differences between the two treatments (two-sided, **P < 0.01). **e** Immunoblotting analysis of total protein extracts from leaves co-infiltrated with 6K2-GFP and buffer (−), empty vector (Vector), or Myc-NbBeclin1. Antibodies against GFP or Myc were used as a primary antibody. **f, g** The effect of the autophagy inhibitor 3-MA on the NbBeclin1-mediated degradation of NbIb-YFP or TuMV-CFP-Nib. Total proteins were isolated from plant leaves agroinfiltrated with NbIb-YFP alone or with Myc-NbBeclin1 (f) or TuMV-CFP-Nib followed by Myc-NbBeclin1 (g) as a primary antibody. **h, i** The effect of silencing of NbATG8a on the NbBeclin1-mediated degradation of NbIb-YFP or CFP-Nib. Plants inoculated with TRV-GUS or TRV-NbATG8a at 14 dpi were agroinfiltrated with NbIb-YFP alone or with Myc-NbBeclin1 (h) or TuMV-CFP-Nib alone or with Myc-NbBeclin1 (i). Total protein was extracted from infiltrated leaves at 3 dpi. Immunoblotting was performed using GFP or Myc antibodies. All immunoblotting assays in this figure were repeated at least three times, and one representative blot was shown. CBB staining of Rubisco large subunit serves as a loading control.
Fig. 6 The GDD motif of Nb is required for the NbBeclin1–Nb interaction and NbBeclin1, via its AIM, interacts with NbATG8a to facilitate the formation of autophagosomes. a Y2H assays to detect possible interactions between NbBeclin1 truncated proteins (NbBeclin1-N and NbBeclin1-C) and Nb and between NbBeclin1 and Nb truncated proteins (Nb-N, Nb-M, and Nb-C). b Y2H assays to detect possible interactions between NbBeclin1 and NbATG8f or NbATG8a and between NbATG8a and NbBeclin1-N, NbBeclin1-C, or NbBeclin1-N AIM mutant (NbBeclin1-ΔAIM). c BiFC assays in H2B-RFP (red) transgenic N. benthamiana leaves at 48 hpi. Yellow fluorescence (green) was observed as a consequence of the complementation of the YN and YC tagged with NbBeclin1 and NbATG8a or NbBeclin1-N and NbATG8a. Bars, 50 μm. d Representative TEM images from N. benthamiana leaf cells agroinfiltrated with buffer (mock), NbBeclin1, NbBeclin1-ΔAIM, NbBeclin1-N, NbBeclin1-N-ΔAIM, or NbBeclin1-C at 60 hpi. Typical autophagcic structures (red arrows) were observed in NbBeclin1- or NbBeclin1-N-expressing leaves in the cytoplasm. Cp chloroplast, CW cell wall, S starch, V vacuole. Bars mean 1 μm or 2 μm as indicated. e The number of typical double-membrane autophagic structures in mock, NbBeclin1-, NbBeclin1-ΔAIM-, NbBeclin1-N-, NbBeclin1-N-ΔAIM-, or NbBeclin1-C-infiltrated leaves. Experiments were repeated three times and typical autophagic structures were counted in 20 cells in each treatment. Values represent the mean number of autophagosomes ±SD per 10 cells. Single asterisk indicates statistically significant difference (P < 0.05) between mock and NbBeclin1-infiltrated leaves, and double asterisks indicate P < 0.01 between mock and NbBeclin1-N (Student’s t-test, two-sided). f Confocal micrographs showing N. benthamiana leaf cells co-infiltrated with Agrobacterium harboring a YFP-NbATG8a expression construct and Agrobacterium carrying NbBeclin1, NbBeclin1-ΔAIM, NbBeclin1-N, NbBeclin1-N-ΔAIM or NbBeclin1-C at 48 hpi. Bars, 25 μm.
tested for the interaction with Nlb by Y2H assays. As shown in Fig. 7f, the Nlb-binding motif was mapped to the N-terminal region (C1) of NbBeclin1-C (strong interaction) or the N-terminal half (C4) of C1 (weak interaction). As expected, both C1 and C4 were unable to mediate Nlb degradation (Fig. 7g). Overexpression of C1 or C4 rather than other NbBeclin1-C fragments inhibited viral replication (Fig. 7e), suggesting that NbBeclin1 binding to the RdRp domain of Nlb suppresses RdRp activity in an autophagy-independent manner.

**Beclin1 and ATG8a negatively regulate TuMV infection.** Given that NbBeclin1-mediated Nlb degradation requires NbATG8a, and NbBeclin1 inhibits RdRp activity by binding to the GDD motif, we next examined the effect of silencing NbBeclin1 or NbATG8a on viral replication. *N. benthamiana* plants were pre-inoculated with TRV-NbBeclin1 (to silence NbBeclin1), TRV-NbATG8a (to silence NbATG8a), or TRV-GUS (as a control) for 7 days and then agroinfiltrated with TuMV-GFP. GFP fluorescence in the inoculated leaves (at 3 dpi) and GFP progression in

![Diagram](attachment:image.png)
the upper non-inoculated leaves (at 6 and 30 dpi) were clearly enhanced in the NbBeclin1- or NbATG8a-silenced plants, compared to controls (Fig. 8a and Supplementary Fig. 12). In concordance, higher levels of TuMV genomic RNA were found in the NbBeclin1- or NbATG8a-silenced plants compared to controls (Fig. 8b). Protoplast transfection assays obtained similar results (Fig. 8c). In contrast, silencing of NbATG8f did not significantly affect TuMV infection (Supplementary Fig. 13). Overall, these results suggest that NbATG8a, and not NbATG8f, is required for NbBeclin1-mediated anti-TuMV defense.

To test whether this antiviral mechanism operates in another plant species, AtATG6, the ortholog of NbBeclin1 in Arabidopsis, was cloned. Y2H and BiFC analyses showed strong interactions between NbIb and AtATG6 (Supplementary Fig. 14a,b). We obtained an atg6 heterozygous mutant (SALK_109281) from the Arabidopsis Biological Resource Center (ABRC) and generated AtATG6 overexpression transgenic Arabidopsis plants (35S:Myc-AtATG6) (Supplementary Fig. 15) for a TuMV infection assay. Compared to TuMV-infected wild-type Col-0, which showed typical symptoms as described39,40, the symptoms in 35S: AtATG6 were delayed for 3–4 days and were attenuated with mild chlorosis and slight stunting (Supplementary Fig. 14c). In contrast, atg6 plants showed more severe symptoms, including leaf necrosis and severe stunting (Supplementary Fig. 14d). Similar results were obtained from the protoplast transfection assay (Supplementary Fig. 14e). We also obtained an Arabidopsis atg8a knockout mutant from ABRC and generated AtATG8a overexpression lines. Consistently, we found that knockout of AtATG8a enhanced viral RNA replication and symptom development, while overexpression of AtATG8a suppressed viral infection (Supplementary Fig. 16).

**NbBeclin1 targets other viral RdRsps to restrict RNA viruses.**

The conserved GDD motif is present in almost all RdRps of viruses other than potyviruses, we cloned RdRps from Cucumber green mottle mosaic virus (CGMMV) of the Tobamovirus genus, and Pepino mosaic virus (PepMV) of the Potexvirus genus (Fig. 9a, b). To address whether NbBeclin1 also targets viral RdRps of viruses other than potyviruses, we cloned RdRps from Cucumber green mottle mosaic virus (CGMMV) of the Tobamovirus genus, and Pepino mosaic virus (PepMV) of the Potexvirus genus (Fig. 9a, b). In both cases, the interacting domain was mapped to the GDD-containing RdRp2 domain and deletion of the GDD motif abolished the interaction (Fig. 9c). Co-IP and BiFC confirmed the data obtained from the Y2H assay (Fig. 9d, e).

The TRV VIGS vector was used to silence NbBeclin1- and the NbBeclin1-silenced plants were challenged with CGMMV and PepMV. The increased viral RNA levels of CGMMV or PepMV were detected in the inoculated leaves at 3 dpi and the upper systemically infected leaves at 14 dpi (Fig. 9f). Consistently, the NbBeclin1-silenced plants showed enhanced susceptibility to CGMMV and PepMV with more severe symptoms (Fig. 9g, h).

**Discussion**

Successful viral infection results from molecular interplays between the invading virus and the host41–44. Viral infection activates autophagy, as shown during infection by many animal viruses15–19,45–46, and activated autophagy selectively degrades viral proteins as an important part of host immunity. A similar picture is also emerging in plants. As mentioned earlier, two recent publications have provided strong evidence that two ATGs, i.e., NBR1 and ATG8 can function as cargo receptors for viral proteins.
proteins to selectively interact with viral proteins to mediate their degradation and defend infection from two DNA viruses. In this study, we found that infection by TuMV, a plant RNA virus, also significantly upregulated the autophagy pathway in plants (Fig. 1 and Supplementary Fig. 1). A TuMV RNA replication mutant, TuMV-ΔGDD, which could express all TuMV proteins including the replication-defective Nb, failed to activate autophagy and induce the high expression of ATGs (Fig. 1). Transient overexpression of individual potyviral proteins alone, including the wild-type Nb, failed to induce autophagy (Supplementary Fig. 2). These data led us to suggest that autophagy induction is likely triggered by the viral replication process or viral RNA accumulation rather than individual potyviral proteins. We further found that Beclin1 interacted exclusively with Nb to mediate Nb degradation likely via the autophagy pathway (Figs. 2–5 and 7 and Supplementary Figs. 5–7, 11). Silencing of Beclin1 enhanced viral replication and symptom development, whereas overexpression of Beclin1 suppressed viral infection (Fig. 8). These data raise a possibility that Beclin1 is a selective receptor against TuMV infection. As a core component of the class III PI3K complex, Beclin1 may regulate autophagy by serving as a scaffold or interaction hub for interacting with diverse protein partners. Genetic lesion studies demonstrate that Beclin1/ATG6 is a conserved requirement in autophagy in plants, humans, and other eukaryotes. Consistent with this study, in their seminal work, Liu and colleagues reported that silencing of Beclin1 and several other ATGs enhances infection by TMV and autophagy is required for the timely HR to restrict TMV infection. It is possible that Beclin1-mediated autophagy inhibits TMV infection via the interaction of Beclin1 and TMV RdRp, and autophagy regulates HR by a different mechanism. In mammalian cells, several viral proteins interact with Beclin1, which has been suggested to be an autophagy counteracting mechanism to promote viral infection. It is not clear if any autophagy-mediated degradation also occurs on these Beclin1-interacting viral proteins.

Viral RdRp is absolutely required for positive-sense RNA virus replication and all viral RdRps have a conserved GDD motif. We found that Beclin1 interacted with the RdRp domain of RdRps from all six test RNA viruses and the highly conserved motif GDD was essential for the interaction (Figs. 2, 6, and 9; Supplementary Fig. 8). Overexpression of the partial NbBeclin1-C fragments (C1 or C4), losing the ability to mediate autophagy-dependent Nb degradation but competent for the interaction with GDD, inhibited TuMV replication (Fig. 7e), suggesting that Beclin1 binding to viral RdRp not only mediates its degradation but also suppresses its replication activity independent of Beclin1-mediated degradation.

ATG8 is a ubiquitin-like protein central to the autophagy pathway by binding to numerous cargo receptors and decorating autophagosomes. In this study, we found that Beclin1 interacted with ATG8a but not ATG8f. Beclin1-mediated Nb degradation required ATG8a (Figs. 3–5; Supplementary Figs. 7 and 9), and the Beclin1-Nb complex or the Beclin1-Nb-VRC complex were co-localized with autophagosomes (Figs. 3 and 4). Beclin1 interacted with ATG8a using Beclin1’s AIM motif at the N terminus (Fig. 6b, c and Supplementary Fig. 9) and with Nb at its C-terminal region (Fig. 6a and Supplementary Fig. 8e–f). Thus it is very attempting to suggest that Beclin1 is a bridge to guide Nb to autophagosomes for degradation. Moreover, the AIM motif of Beclin1 is required for its interaction with the viral RdRp.
Beclin1 was necessary for the formation of autophagosomes and induction of autophagy flux (Fig. 6d–f and Supplementary Figure 11a–c), supporting Beclin1 may play roles as an autophagy substrate and a functional receptor.

Silencing and overexpression of ATG8a affected plant susceptibility to infection in the same ways as Beclin1 (Supplementary Fig. 16). These findings revealed the biological importance of ATG8a in Beclin1-mediated selective autophagy in suppressing viral infection. However, knockdown of ATG8f did not significantly affect Beclin1-mediated Nb degradation and viral infection (Supplementary Figs 7 and 13). The fact that Beclin1 binds to NbATG8a rather than NbATG8f suggests that ATG8 family proteins may selectively bind to different autophagy receptors.
In addition to ATG8, we also examined the possible involvement of several other core autophagy proteins. Silencing or knockdown of PI3K or VPS15 promoted TuMV infection in *N. benthamiana* or Arabidopsis plants (Supplementary Fig. 17), which is not surprising as PI3K and VPS15, together with Beclin1, form the PI3K complex that initiates autophagy15,16. Knockout of another core autophagy gene ATG2, known to be involved in the early steps of autophago-some biogenesis, also increased plant susceptibility to TuMV infection (Supplementary Figs 13 and 18). In contrast, silencing of ATG5 or ATG7 did not affect viral infection (Supplementary Figs 13 and 18). We further found that it is not ATG5/ATG7 but ATG2 that is essential for the functionality of Beclin1-mediated degradation of Nib (Supplementary Fig. 7). The fact that NbBeclin1-mediated Nib degradation is dependent on ATG2 and independent of ATG5/ATG7 leads us to suggest that there is an alternative autophagy pathway in plants. In mammalian cells, it has been shown that ATG5 or ATG7 deficiency fails to block the formation of autophagosomes/autolysosomes for autophagy-mediated protein degradation upon induction by certain stressors, and thus macro-autophagy can take place via at least two pathways: an ATG5/ATG7-dependent conventional pathway and an ATG5/ATG7-independent non-canonical pathway55,56. Our data raise the possibility that this might also hold true for plants and further study is needed to clarify this non-canonical pathway in plants.

Supporting the concept of the antiviral role of autophagy in potyviral infection, Nakahara and colleagues showed that the calmodulin-like protein in *N. tabacum*, rgs-CaM, counterattacks TEV HC-Pro and other VSRs by binding to the dsRNA-binding domain to inhibit its RNAi capacity and the resulting interaction complex is degraded by the autophagy pathway to enhance host antiviral defense56. Conversely, rgs-CaM in *N. benthamiana* suppresses RNA silencing and promotes geminivirus infection by autophagy-dependent degradation of suppressor of gene silencing 3 (SGS3), a key component of the RNA silencing pathway57,58. Recently, we also found that Vpg, the second potyviral VSR, interacts with SGS3 and mediates the degradation of SGS3 and its intimate partner RNA-dependent RNA polymerase 6 (RDR6) via the ubiquitin–proteasome and autophagy pathways, suggesting a possible mechanism by which VPG sabotages host antiviral RNA silencing to promote viral infection58. The proviral role of autophagy has also been demonstrated during viral infection in animals15. For example, knockout of ATG7 or Beclin1 inhibits the replication of *Hepatitis C virus* (HCV), demonstrating that they are proviral factors in HCV infection59,60. These data suggest that, during the co-evolutionary arms race, viruses have also developed strategies to subvert autophagy for their own benefit. Obviously, this increases the complexity of outcomes of induced autophagy in response to viral infection.

Based on the discussion above, we propose a model summarizing the possible role of autophagy in potyviral infection (Fig. 10). Viral infection activates autophagy, leading to the accumulation of high levels of ATGs. Beclin1 interacts with Nib at the GDD motif and directly suppresses its RdRp activity to restrict viral infection. The Beclin1–Nib complex is targeted for autophagic degradation possibly through the interaction of Beclin1 and ATG8a via AIP. In addition, the activated autophagy pathway also targets two VSRs, e.g., HC-Pro and Vpg53,57. As an antiviral mechanism, the former is recognized by rgs-CaM to induce autophagy-dependent degradation56. The latter binds to SGS3 to mediate the degradation of SGS3 and RDR6 via autophagy, which promotes viral infection57. The mechanism(s) by which autophagy is recruited is not clear. Based on this study, targeting viral RdRp by Beclin1 is likely a general antiviral mechanism in plants. This opens up a novel exciting avenue for the control of plant RNA viruses through upregulation of Beclin1 and/or ATG8a or overexpression of the GDD-binding domain of Beclin1. It would be interesting to determine whether Beclin1 also interacts with RdRps of RNA viruses in mammalian cells and confers broad-spectrum resistance to viral infection therein.

**Methods**

### Viral strains

The recombinant viruses TuMV, TuMV-GFP, TuMV-AGD, TuMV-GFP-Nb-6K2-mCherry, TuMV-6K2-mCherry, and PPV-GFP-mCherry were previously described56,57,58, and PPV and CGMMV were isolated from tomato and cucumber in a greenhouse in Ontario, Canada.

### Plasmid construction

GenBank accession numbers of genes and viral sequences analyzed in this study are as follows: *NbBeclin1* (AY701316), *NbPIK3* (KX120977), *NbVPS15* (KU561371), *NbATG2* (KU561373), *NbATG3* (KX369396), *NbATG5* (KX369397), *NbATG7* (KX369398), *NbATG8a* (KX120976), *NbATG9* (KU561372), *AtATG6* (NM_202746), TuMV (KX369395), SMV (KU717224), PPV (KX998124), N. benthamiana (*AtActin* (AY179085), and *AtAc- tin1* (AT3G18780). Gateway technology (Invitrogen, Burlington, Ontario, Canada) was used to generate all the plasmid constructs used in this study, unless otherwise stated. Gene sequences were amplified by PCR using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Pickering, ON, Canada) for cloning purposes. GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA) was employed for regular PCR analysis. The full-length TuMV P1, HC-Pro, P3, PJN-PIPO, 6K1, CI, 6K2, Vpg, NlA-Pro, Nib and CP, PPV Nib, SMV Nib, and TEV Nib were cloned previously63,64,65,66. *NbBeclin1*, *NbATG8a*, and *NbATG8f*-coding sequence were generated from *cDNA* derived from *N. benthamiana* leaves and *AtATG6*-coding sequence was generated from *cDNA* derived from Arabidopsis leaves (primers are listed in Supplementary Table 1). The GDD mutants of TuMV Nib and PPV Nib

**Fig. 9** NbBeclin1 targets RdRps of plant viruses distinct to potyviruses and inhibits viral infection. a, b Schematic representation of the full-length CGMMV RdRp (a) and PepMV RdRp (b). The positions of the first and last amino acid residues of each conserved domain are indicated. Met methyltransferase domain, Hel helicase domain, Rdrp2 Rdrp2 domain. c NbBeclin1 interacts with CGMMV (C) and PepMV (Pe) RdRps or their Rdrp2 domains but not with their GDD mutants or other domains, i.e., Met and Hel in the Y2H assays. d Co-IP analysis of possible interactions of NbBeclin1 with different domains or the GDD mutant of CGMMV and PepMV RdRps in plants. *N. benthamiana* leaves were agroinfiltrated with CGMMV and PepMV RdRps in plants. e, f Quantification of CGMMV or PepMV RNA levels by qRT-PCR. The plants were pre-inoculated with buffer (mock), TRV1+TRV2-GUS, or TRV1+TRV2-NbBeclin1 for 7 days. RNA was extracted from CGMMV or PepMV-inoculated or systemically infected leaves at 3 dpi and 14 dpi, respectively. The values are presented as means of fold change ±SD relative to mock-treated plants. Error bars represent SD. Three independent experiments, each consisting of three biological replicates, were carried out. Values from one representative result were used to plot a histogram and were normalized with *NbActin* as the internal reference. The data were analyzed using Student’s t-test and double asterisks denote significant differences compared to the CGMMV- or PepMV-infected NbBeclin1-silenced plants from control plants pretreated with mock (two-sided, "*P* < 0.01). g, h Symptoms of CGMMV (g) or PepMV (h) infected plants at 21 dpi. The plants were pre-inoculated with buffer (mock), TRV-GUS, or TRV-NbBeclin1 for 14 days. Mock inoculated with buffer, CGMMV inoculated with CGMMV, PepMV inoculated with PepMV.
were cloned from the infectious clone TuMV-ΔGDD\(^36\) and PPV-ΔGDD\(^36\). Coding cDNA sequences of RdRp and its domains Met, Hel, and RdRp2 of CGMMV (GenBank accession: AB015146) and PepMV (KY031324) were cloned by RT-PCR. CGMMV RdRp2-ΔGDD, PepMV RdRp2-ΔGDD, TEV Nb-ΔGDD, and SMV Nb-ΔGDD were cloned by overlapping PCR using the specific primers (Supplementary Table 1). One AIM that matches the consensus amino acid sequence X-3X-2X-1-W/F/Y-X1X2/L/V was located in the N-terminal of NbBeclin1: EESFVVL. The mutant of AIM motif (NbBeclin1-N\(^\Delta\)AIM) was produced by replacing EESAVV with EESAVFA using a high-stringency selective medium lacking tryptophan, leucine, and histidine (SD/-Trp-Leu-His-Ade) to analyze the interaction. In brief, yeast cells (strain Y2H Gold, Clontech catalog 

**Fig. 10** Proposed model for the possible roles of autophagy in the potyviral infection. Infection by positive-sense RNA viruses activates autophagy in plant cells. Beclin1 (ATG6) as well as Beclin1 that is targeted to the GDD motif of the viral RNA-dependent RNA polymerase (RdRp) to inhibit virus replication, which is independent of the autophagy pathway. Beclin1 may also serve as a cargo receptor to interact with the viral RdRp and target the RdRp-containing virus replication complex via the interaction of Beclin1 with other autophagy proteins (e.g., ATG8a) to autophagosomes for degradation. The mechanistic details leading to the autophagy-mediated degradation remain to be fully understood. Simultaneously, HC-Pro, a virulence factor and the major potyviral VSR, is hijacked by host rgs-CaM via the autophagy pathway. Beclin1 may also serve as a cargo receptor to interact with the viral RdRp and target the RdRp-containing virus replication complex via the autophagy pathway\(^56\). Simultaneously, the second potyviral VSR, VPg, mediates the degradation of RNA silencing components SG3 and RDR6 via the autophagy pathway to suppress antiviral RNA silencing\(^58\).
Protoplast isolation and TuMV replication assay. Mesophyll protoplasts were isolated and transfected essentially as described.6 In brief, mesophyll protoplasts were prepared from 4-week-old wild-type, caf (SALK_109281), SALK_045344c, 35S:Myc-AtATG6a, 35S:Myc-AtATG6b (SALK_045344c), or 35S:Myc-AtATG8a Arabidopsis leaves or TRV-GUS-, TRV-NbBeclin1-, and TRV-NbATG8s-silenced newly emerged N. benthamiana leaves as described previously.7 About 1×10⁶ protoplasts were transfected with 20 μg pcambia2300-TuMV-GFP plasmid in 0.5% PEG 6000 in 0.8 M mannitol and 1 M CaCl₂ at room temperature for 20 min. Transformed protoplasts were then washed and resuspended in W5 buffer (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, and 2 mM MES, pH 5.7) and incubated for the virus replication. At 16, 24, and 48 h posttransfection, the protoplasts were harvested for the extraction of RNA. The protoplast transfection assay was repeated at least three times, and one representative result was used to plot a histogram.

RNA extraction and qRT-PCR analysis. Total RNA was extracted from mock (Agrobacterium-carrying empty vector), TuMV-ΔGDD-, or TuMV-ΔGDD-infected N. benthamiana leaves or Agrobacterium-carrying empty vector (Vec), Nib-YFP, Mcc-NbBelin1 or Mcc-NbBeclin1 and Nib-YFP-infected N. benthamiana leaves or TRV-VIGS-treated N. benthamiana leaves or TuMV-GFP, CGMMV-, or PeyMV-infected TRV-VIGS-treated N. benthamiana leaves or mock, TuMV-ΔGDD, TuMV, TuMV-GFP, CGMMV- or PeyMV systemically infected Arabidopsis, N. benthamiana, or TRV-VIGS-treated N. benthamiana leaves at different time periods using the RNeasy Plant Mini Kit (Qiagen) and treated with DNase I following the manufacturer’s instructions. cDNA synthesized from reverse transcription of RNA samples was used to determine the mRNA expression levels of target genes as well as quantifying TuMV accumulation levels using primers indicated. NActin or ActinII was used as an internal control for N. benthamiana and Arabidopsis, respectively. First-strand cDNA was synthesized from 1 μg total RNA using Oligo(dT)12–18 primer or specific primers and SuperScript® III reverse transcriptase (Invitrogen) following the recommended protocol. A list of primers used in this study is provided (Supplementary Table 1). qRT-PCR was conducted and analyzed as described previously.7,9

Chemical treatments and TEM. Phosphate-buffered saline containing dimethyl sulfoxide (DMSO; control) or an equal volume of DMSO with 5 mM 3-MA, 100 μM Con A (Sigma) for inhibition of autophagy was infiltrated into leaves 8–12 h before samples were collected. For TEM observation, detailed information has been described previously.10 Upper non-inoculated leaves of N. benthamiana plants infected with buffer (mock), TuMV, and TuMV-ΔGDD at 7 dpi or agro-infected N. benthamiana leaves with mock, NbBeclin1, NbBeclin1 (ΔAIM), NbBeclin1-N, NbBeclin1-NAIM, or NbBeclin1-C at 60 hpi were pretreated with 10 mM 3-MA for 8 h and then were cut into small pieces (1×4 mm²). The treatments and the examination of the sampled tissues were performed as described.11

Immunoblotting and immunoprecipitation. Total protein was extracted from infiltrated leaf patches or mock, TuMV-, and TuMV-ΔGDD-infected N. benthamiana leaves as described previously.12 Western blotting was performed with primary antibodies and secondary antibodies and visualized procedure. Western blots were scanned using Discovery™ Imaging System, and densitometric analysis was performed with ImageJ software according to the manufacturer’s protocol (ECL; GE Healthcare). Immunoprecipitation was done on protein extracts from N. benthamiana leaves at 2 dpi by using GFP-Trap® MA (Chromotek) according to the manufacturer’s instructions with some modifications. Briefly, about 1 g leaf tissue were ground in liquid nitrogen and extracted with 2 ml lysis buffer (10% glycerol, 25 mM Tris HCl (pH 7.5), 1% EDTA, 150 mM NaCl, 2% w/v PVPP, 100 mM DTT, 1× EDTA-free protease inhibitor cocktail (Roche), 0.1% Igepal CA-630 (Sigma)). After incubation on ice for 30 min with gentle shaking, the mixtures were centrifuged at 3000 g at 4 °C for 10 min. The supernatants were then centrifuged with full speed at 4 °C for 10 min. Extracts were passed through a 0.45 μm filter and then incubated with GFP-Trap® MA beads (Chromotek) for 3 h at 4 °C with gentle shaking. The precipitations were washed four times with ice-cold immunoprecipitation buffer (10% glycerol, 25 mM Tris HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1× EDTA-free protease inhibitor cocktail (Roche), 0.1% Igepal CA-630) at 4 °C and analyzed by immunoblotting with Myc, HA, or GFP antibodies. Uncropped images of immunoblots are shown in Supplementary Figs. 19 through 26.

Data availability. The authors declare that all data supporting the findings of this study are available in the manuscript and its Supplementary Information files or are available from the corresponding author upon request.

Received: 20 April 2017 Accepted: 28 February 2018
Published online: 28 March 2018

References
1. Mizushima, N., Yoshimori, T. & Ohsumi, Y. The role of Atg proteins in autophagosome formation. Annu. Rev. Cell Dev. Biol. 27, 107–132 (2011).
2. Lamm, K. A., Yoshimori, T. & Toome, S. A. The autophagosome: origins unknown, biogenesis complex. Nat. Rev. Mol. Cell Biol. 14, 759–774 (2013).
3. Boya, P., Reggiori, F. & Codogno, P. Emerging regulation and functions of autophagy. Nat. Cell Biol. 15, 713–720 (2013).
4. Üstün, S., Hafren, A. & Hofius, D. Autophagy as a mediator of life and death in plants. Curr. Opin. Plant Biol. 40, 122–130 (2017).
5. Keller, R., De la Concepcion, I. C., Mapked, A., Kamoun, S. & Daggas, Y. D. ATG8 expansion: a driver of selective autophagy diversification? Trends Plant Sci. 22, 204–214 (2016).
6. Feng, Y., Yao, Z. & Kleinschmidt, D. J. How to control self-digestion: transcriptional, post-transcriptional, and post-translational regulation of autophagy. Trends Cell Biol. 25, 354–363 (2015).
7. Khaminets, A., Behl, C. & Dikie, I. Ubiquitin-dependent and independent signals in selective autophagy. Trends Cell Biol. 26, 6–16 (2016).
8. Wen, X. & Klionsky, D. J. An overview of macroautophagy in yeast. J. Mol. Biol. 428, 1681–1699 (2016).
9. Li, P. & Vierstra, R. D. Autophagy: a multifaceted intracellular system for bulk and selective recycling. Trends Plant Sci. 17, 526–537 (2012).
10. Hofius, D., Li, L., Hafren, A. & Coll, N. S. Autophagy as an emerging arena for plant-pathogen interactions. Curr. Opin. Plant Biol. 38, 117–123 (2017).
11. Chen, L. & Dikic, I. Autophagy in antimicrobial immunity. Mol. Cell 54, 224–233 (2014).
12. Shelly, S., Lukinova, N., Bambina, S., Berman, A. & Cherry, S. Autophagy is an essential component of Drosophila immunity against vesicular stomatitis virus. Immunity 30, 588–599 (2009).
13. Orvedahl, A. et al. Autophagy protects against sindbis virus infection of the central nervous system. Cell 161, 115–127 (2015).
14. Liu, Y. et al. Autophagy regulates programmed cell death during the plant innate immune response. Cell 121, 567–577 (2005).
15. Hafren, A. et al. Selective autophagy limits cauliflower mosaic virus infection by NBR1-mediated targeting of viral capsid protein and particles. Proc. Natl. Acad. Sci. USA 114, E2026–E2035 (2017).
16. Haxim, Y. et al. Autophagy functions as an antiviral mechanism against geminivirus infections in plants. Nat. f. 6, e23897 (2017).
17. Clavel, M., Michaeli, S. & Genschik, P. Autophagy: a double-edged sword to fight plant viruses. Trends Plant Sci. 22, 646–648 (2017).
18. Revers, F. & Garcia, J. A. Molecular biology of potyviruses. Adv. Virus Res. 89, 101–199 (2015).
19. Kamoun, S. & Daggas, D. Y. ATG8 expansion: a driver of selective autophagy diversification? Trends Plant Sci. 22, 204–214 (2016).
20. Clavel, M., Michaeli, S. & Genschik, P. Autophagy: a double-edged sword to fight plant viruses. Trends Plant Sci. 22, 646–648 (2017).
21. Feuerherd, M. & Grams, C. J. Molecular biology of potyviruses. Adv. Virus Res. 89, 101–199 (2015).
22. Yoshimoto, K. et al. Processing of ATG8s, ubiquitin-like proteins, and their conjugates. NATURE COMMUNICATIONS | DOI: 10.1038/s41467-018-03658-2 | www.nature.com/naturecommunications
32. Wang, L., Tian, Y. & Ou, J. H. Hepatitis C virus promotes viral infection by interacting with the host NFR1- mediated immune response. Plant Cell 29, 508–525 (2017).

33. Fujiki, Y., Kohki, Y. & Yoshinori, O. An Arabidopsis homolog of yeast ATG6/VPS30 is essential for pollen germination. Plant Physiol. 143, 1132–1139 (2007).

34. Cheng, X., Deng, P., Cui, H. & Wang, A. Visualizing double-stranded RNA distribution and dynamics in living cells by dsRNA binding-dependent fluorescence complementation. Virology 485, 439–451 (2015).

35. Cui, H. & Wang, A. Plasmovirus virus 6K1 protein is required for viral replication and targets the viral replication complex at the early stage of infection. J. Virol. 90, 5119–5131 (2016).

36. Zientara-Rytter, K. et al. Identification and functional analysis of Joka2, a tobacco member of the family of selective autophagy cargo receptors. Autophagy 7, 1145–1158 (2011).

37. Kalvari, I. et al. LIR: A web resource for prediction of Atg9-family interacting proteins. Autophagy 10, 913–925 (2014).

38. Lellis, A.D., Kasschau, K.D., Whitham, S.A. & Carrington, J.C. Loss-of-susceptibility mutants of Arabidopsis thaliana reveal an essential role for elf(iso)4E during potyvirus infection. Curr. Biol. 12, 1046–1051 (2002).

39. Huang, T. S., Wei, T., Laliberté, J. F. & Wang, A. A host RNA helicase-like protein, AtRHH, interacts with the potyviral genome-linked protein, VPg, associates with the virus accumulation complex, and is essential for infection. Plant Physiol. 152, 255–266 (2010).

40. den Boon, J. A., Diaz, A. & Ahlquist, P. Cytoplasmic viral replication complexes. Trends Cell Biol. 6, 301–307 (1996).

41. Huang, T. S., Wei, T., Laliberté, J. F. & Wang, A. A host RNA helicase-like protein, AtRHH, interacts with the potyviral genome-linked protein, VPg, associates with the virus accumulation complex, and is essential for infection. Plant Physiol. 152, 255–266 (2010).

42. Nagy, P. D. & Pogany, J. The dependence of viral RNA replication on co-orthostress factors. Nat. Rev. Microbiol. 10, 137–149 (2012).

43. Garcia, J. A. & Pallás, V. Viral factors involved in plant pathogenesis. Curr. Opin. Virol. 11, 21–30 (2015).

44. Wang, A. Dissecting the molecular network of virus-plant interactions: the complex roles of host factors. Nat. Rev. Microbiol. 10, 137–149 (2012).

45. Garcia, J. A. & Pallás, V. Viral factors involved in plant pathogenesis. Curr. Opin. Virol. 11, 21–30 (2015).

46. Wang, A. Dissecting the molecular network of virus-plant interactions: the complex roles of host factors. Annu. Rev. Phytopathol. 53, 45–66 (2015).

47. Hansen, M. D. et al Hepatitis C virus triggers Golgi fragmentation and virus-induced vesicles with chloroplasts. PLoS Pathog. 9, e1003378 (2013).

48. Orvedahl, A. et al. HSV-1 ICP34.5 confers neurovirulence by targeting the protein, AtRH8, interacts with the potyviral genome-linked protein, VPg, associates with the virus accumulation complex, and is essential for infection. Plant Physiol. 152, 255–266 (2010).

49. Koonin, E. V. & Dolja, V. V. Evolution and taxonomy of positive strand RNA virus replication complexes. J. Virol. 83, 10460–10471 (2009).

50. Wei, T. et al. Formation of complexes at plasmodesmata for potyvirus intercellular movement is mediated by the viral protein P3N-PIPO. PLoS Pathog. 6, e1000962 (2010).

51. Wei, T., Zhang, C., Hou, X., Sanfacon, H. & Wang, A. The SNARE protein Syt7 is essential for turnip mosaic virus infection by mediating fusion of virus-induced virus-induced vesicles with chloroplasts. PLoS Pathog. 9, e1003378 (2013).

52. Karimi, M., Inzé, D. & Depicker, A. GATEWAY® vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci. 7, 193–195 (2002).

53. Lu, Q. et al. Arabidopsis homolog of the yeast TREX-2 mRNA export complex: components and anchoring nucleoprotein. Plant J. 61, 259–270 (2010).

54. Earley, K. W. et al. Gateway-compatible vectors for plant functional genomics and proteomics. Plant J. 45, 616–626 (2009).

55. Zhu, H. et al. Arabidopsis Argonaute10 specifically sequesters miR166/165 to regulate shoot apical meristem development. Cell 145, 242–256 (2011).

56. Deng, P., Wu, Z. & Wang, A. (2015). The multifunctional protein CI of potyviruses plays interlinked and distinct roles in viral genome replication and intercellular movement. Virol. J. 12, 141 (2015).

57. Li, F., Huang, C., Li, Z. & Zhou, X. Suppression of RNA silencing by a plant RNA virus satellite requires a host calmodulin-like protein to repress RDR6 expression. PLoS Pathog. 10, e1003921 (2014).

Acknowledgements
We thank Michael M. Goodin (University of Kentucky) for the transgenic H2B:GFP-FFP line; Yule Liu (Tsinghua University) for the TRV VIGS vector; Yuhai Cui [Agricultural and Agri-Food Canada (AAFC)] for the modified gateway vectors of pGADT7, pGBKTK, p35S:YN, and p35S:YCI, Xiaojuan Wu (the Wang lab) for providing the PepMV infectious clone; Xiaofei Cheng and Hongguang Cui (AAFC) for helpful discussion and suggestions; Alex Molnar (AAFC) for photography and artwork; and Jamie McNeil (AAFC) for technical support. This work was supported in part by a GRDI grant and an A-base grant from AAFC and a discovery grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) to A.W.

Author contributions
F.L. and A.W. designed the project. F.L., C.Z., Y.L., and G.W. conducted experiments. All authors analyzed the data and reviewed the manuscript. F.L. and A.W. wrote the paper.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-03658-2.

Competing interests: The authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

60. Tanida, I. et al. Knockdown of autophagy-related gene decreases the production of infectious hepatitis C virus particles. Autophagy 5, 937–945 (2009).

61. Clough, S. J. & Bent, A. F. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16, 735–743 (1998).

62. Cotton, S. et al. Turnip mosaic virus RNA replication complex vesicles are mobile, align with microfilaments, and are each derived from a single viral genome. J. Virol. 83, 10460–10471 (2009).

63. Wei, T. et al. Formation of complexes at plasmodesmata for potyvirus intercellular movement is mediated by the viral protein P3N-PIPO. PLoS Pathog. 9, e1000962 (2010).

64. Wei, T., Zhang, C., Hou, X., Sanfacon, H. & Wang, A. The SNARE protein Syt7 is essential for turnip mosaic virus infection by mediating fusion of virus-induced vesicles with chloroplasts. PLoS Pathog. 9, e1003378 (2013).

65. Karimi, M., Inzé, D. & Depicker, A. GATEWAY® vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci. 7, 193–195 (2002).

66. Lu, Q. et al. Arabidopsis homolog of the yeast TREX-2 mRNA export complex: components and anchoring nucleoprotein. Plant J. 61, 259–270 (2010).

67. Earley, K. W. et al. Gateway-compatible vectors for plant functional genomics and proteomics. Plant J. 45, 616–626 (2009).

68. Zhu, H. et al. Arabidopsis Argonaute10 specifically sequesters miR166/165 to regulate shoot apical meristem development. Cell 145, 242–256 (2011).

69. Deng, P., Wu, Z. & Wang, A. (2015). The multifunctional protein CI of potyviruses plays interlinked and distinct roles in viral genome replication and intercellular movement. Virol. J. 12, 141 (2015).

70. Li, F., Huang, C., Li, Z. & Zhou, X. Suppression of RNA silencing by a plant RNA virus satellite requires a host calmodulin-like protein to repress RDR6 expression. PLoS Pathog. 10, e1003921 (2014).