Autophagy functions as an antiviral mechanism against geminiviruses in plants

Yakupjan Haxim†, Asigul Ismayil†, Qi Jia†, Yan Wang†, Xiying Zheng†, Tianyuan Chen†, Lichao Qian†, Na Liu†, Yunjing Wang†, Shaojie Han†, Jiaxuan Cheng†, Yijun Qi†, Yiguo Hong‡, Yule Liu*†

†These authors contributed equally to this work

Abstract Autophagy is an evolutionarily conserved process that recycles damaged or unwanted cellular components, and has been linked to plant immunity. However, how autophagy contributes to plant immunity is unknown. Here we reported that the plant autophagic machinery targets the virulence factor βC1 of Cotton leaf curl Multan virus (CLCuMuV) for degradation through its interaction with the key autophagy protein ATG8. A V32A mutation in βC1 abolished its interaction with NbATG8f, and virus carrying βC1V32A showed increased symptoms and viral DNA accumulation in plants. Furthermore, silencing of autophagy-related genes ATG5 and ATG7 reduced plant resistance to the DNA viruses CLCuMuV, Tomato yellow leaf curl virus, and Tomato yellow leaf curl China virus, whereas activating autophagy by silencing GAPC genes enhanced plant resistance to viral infection. Thus, autophagy represents a novel anti-pathogenic mechanism that plays an important role in antiviral immunity in plants.

Introduction Plants have evolved various defense mechanisms to combat plant pathogens, including viruses. The two major mechanisms for plant antiviral immunity are RNA silencing and resistance (R) gene-mediated resistance (Mandadi and Scholthof, 2013). RNA silencing is a sequence-specific mechanism used to directly defend host cells against foreign invaders such as viruses and transposable elements (Ding, 2010). By contrast, the activation of R gene-mediated resistance triggers a rapid defense response that often includes localized programmed cell death, known as the hypersensitive response (HR). The HR can prevent local viral infection and elicit systemic acquired resistance to viral infection.

Autophagy is an evolutionarily conserved mechanism that recycles damaged or unwanted cellular materials under stress conditions or during specific developmental processes (Liu and Bassham, 2012), and plays a critical role in multiple physiological processes, including plant biotic stress responses (Han et al., 2011). During the plant’s response to incompatible pathogens, autophagy contributes to HR cell death but restricts the spread of programmed cell death beyond the initial infection site (Liu et al., 2005; Patel and Dinesh-Kumar, 2008; Hofius et al., 2009; Yoshimoto et al., 2009). During compatible plant–pathogen interactions, autophagy positively regulates plant defense responses against necrotrophic pathogens (Lai et al., 2011; Lenz et al., 2011; Kabbage et al., 2013). However, disrupting autophagy in Arabidopsis thaliana leads to enhanced resistance to the biotrophic pathogen powdery mildew and dramatic pathogen-induced cell death (Wang et al., 2011). The role of autophagy in plant defense responses against the bacterial pathogen Pseudomonas syringae DC3000 is controversial (Patel and Dinesh-Kumar, 2008; Hofius et al., 2009; Lenz et al., 2011). However, it is unclear how autophagy links plant immunity in these studies.
Autophagy may link plant immunity in different ways, with autophagy playing a role in degrading pathogen effectors or defense-related plant proteins, or pathogen effectors interfering with autophagy. Indeed, viral proteins are reported to promote autophagic degradation of plant host RNAi-related components (Derrien et al., 2012; Cheng and Wang, 2016). In addition, 2b protein from Cucumber mosaic virus is thought to be targeted for degradation by autophagy through the calmodulin-like protein rgsCaM (Nakahara et al., 2012). Recently, an oomycete effector is reported to interfere with autophagy by depleting the putative selective autophagy cargo receptor Joka2 out of ATG8 complexes (Dagdas et al., 2016). However, the role of autophagy in degrading pathogen effectors or plant defense-related proteins and the effect of viral effectors on autophagy remain uncertain in plants. All current findings are based on the data from chemical autophagy inhibitor treatments (with potential off-target effects) and/or silencing of autophagy-nonspecific autophagy-related (ATG) gene Beclin 1 (Derrien et al., 2012; Nakahara et al., 2012; Cheng and Wang, 2016). Further, in these above studies, no data showed that disruption of classic autophagy really affects pathogen invasion. Moreover, to date, there is no evidence to show that autophagy has a role during any compatible plant-virus interactions.

Geminiviruses are a large, diverse group of plant viruses with circular single-stranded DNA genomes, and many geminiviruses cause devastating diseases in different crops. These viruses often occur in disease complexes. For example, Cotton leaf curl Multan virus (CLCuMuV), in association with the disease-specific satellite DNA Cotton leaf curl Multan betasatellite (CLCuMuB), causes cotton leaf curl disease, a major viral disease in cotton (Sattar et al., 2013). In addition to cotton, CLCuMuV infects many other plants, including Nicotiana benthamiana. CLCuMuV encodes six proteins, namely C1, C2, C3, C4, V1 and V2, whereas CLCuMuB is approximately half the size of the CLCuMuV DNA genome and encodes a single protein, βC1 (Briddon et al., 2003). Like most βC1 factors encoded by geminivirus betasatellites, CLCuMuB βC1 is required by CLCuMuV for the induction of disease symptoms in plants. In addition, CLCuMuB βC1 enhances the accumulation of its helper virus, CLCuMuV (Saeed et al., 2015; Jia et al., 2016), and is involved in RNA silencing (Amin et al., 2011). Recently, we and other groups reported that geminivirus βC1s can subvert ubiquitination to assist their helper viruses to infect plants (Jia et al., 2016; Shen et al., 2016).
Results

CLCuMuB βC1 interacts with the autophagy-related protein ATG8

To investigate the role of CLCuMuB βC1 (hereafter βC1) in plant–virus interactions, we performed yeast two-hybrid screening of a Solanum lycopersicum cDNA library using βC1 as the bait. From this screen, we identified the autophagy-related protein SIATG8f as a βC1-interacting protein. We also found that βC1 interacted with NbATG8f, the closest N. benthamiana homolog of SIATG8f in yeast (Figure 1A).

We validated the in vivo and in vitro interactions between βC1 and NbATG8f using Glutathione S-Transferase (GST) pull-down (Figure 1B) and co-immunoprecipitation assays (Figure 1C). Moreover, we identified an 11-amino acid motif from residue 30 to 40 of βC1 that is necessary for its interaction with NbATG8f (Figure 1—figure supplement 1). Strikingly, GST pull-down and co-immunoprecipitation assays indicated that the mutant protein βC1V32A was unable to interact with NbATG8f (Figure 1B,C). Further, co-immunoprecipitation assays indicated that βC1 also interacted with other three ATG8 isoforms (Figure 1—figure supplement 2).

We then performed a bimolecular fluorescence complementation (BiFC) assay to identify the subcellular localization of the βC1–NbATG8f interaction in plant cells. A positive interaction between nYFP-βC1 and cYFP-NbATG8f was observed in both the cytoplasm and vacuoles of plant cells, as indicated by the presence of yellow fluorescence (Figure 1D). However, no such interaction was detected between nYFP-βC1V32A and cYFP-NbATG8f (Figure 1D), although all constructs were successfully expressed (Figure 1E). We further confirmed the vacuolar localization of the βC1–NbATG8f interaction by performing time-lapse observations of mesophyll cells by confocal microscopy, which revealed Brownian motion of fluorescent punctate structures within the central vacuole (Video 1).

In addition, we found that YFP-βC1, but not YFP-βC1V32A, co-localized with GFP-NbATG8f-positive bodies in both the cytoplasm and central vacuoles of mesophyll cells of N. benthamiana leaf tissue, as revealed by confocal microscopy (Figure 1—figure supplement 3). The vacuolar deposition of YFP-βC1 was also confirmed by time-lapse observations of mesophyll cells by confocal microscopy, which revealed Brownian motion of fluorescent punctate structures within the central vacuole (Video 2), which is consistent with the vacuolar localization of the βC1–NbATG8f interaction (Video 1).

Taken together, these results demonstrate that βC1 specifically interacts with NbATG8s and the V32 residue is essential for the βC1–NbATG8f interaction.

Autophagy is induced during CLCuMuV infection

Since βC1 specifically interacted with NbATG8s, we hypothesized that autophagy affects the infection of plants by geminiviruses. To test this hypothesis, we investigated whether geminivirus infection could induce autophagy in N. benthamiana. First, we performed quantitative RT-PCR (qRT-PCR) analysis, finding that mRNA levels of NbATG2, NbATG3, NbATG5, and NbATG7 were upregulated during the infection of plants with CLCuMuV (CA) plus CLCuMuB (β) (hereafter referred to as CLCuMuV infection or CA+β) (Figure 2—figure supplement 1). We then used Cyan Fluorescent Protein (CFP)-tagged NbATG8f (CFP-NbATG8f) as an autophagosome marker (Han et al., 2015) to visualize possible autophagic activity. In N. benthamiana plants infected with CA+β, we observed increased numbers of autophagosomes (represented by CFP-NbATG8f puncta) (Figure 2A,B). Transmission electron microscopy confirmed that viral infection increased the number of autophagic structures in infected cells (Figure 2C,D) compared to the control. Further, we tested the effect of CLCuMuV infection on autophagy flux using Joka2/NBR1, a selective autophagy cargo receptor, as a protein marker. Joka2 has been used as a useful tool to measure autophagy flux in plants because it is degraded in the vacuole once autophagy activity increases (Zhou et al., 2013; Xu et al., 2017). Indeed, we found that CLCuMuV infection (CA+β) reduced the protein level of NbJoka2, although it did not change mRNA level of NbJoka2 (Figure 2—figure supplement 2), indicating that viral infection enhances autophagic flux. These data demonstrate that CLCuMuV infection induces autophagy.
Figure 1. CLCuMub βC1 interacts with NbATG8f in vivo and in vitro. (A) βC1 interacts with NbATG8f in yeast. SKY48 yeast strains containing AD-NbATG8f transformed with BD-βC1 or BD (control) were grown on Leu- selection plates at 28°C for 4 d. The positive interaction was indicated by the blue colony formation on X-gal-containing galactose (Gala) and raffinose (Raf) but not on plates containing glucose (Glu). (B) GST pull-down assay to show the in vitro interaction of NbATG8f with βC1, but not βC1V32A. The total soluble proteins of E. coli expressing NbATG8f-6xHis were incubated with GST-βC1 or GST-βC1V32A immobilized on glutathione-sepharose beads and monitored by anti-His antibody. (C) βC1 was co-immunoprecipitated with NbATG8f. GFP-NbATG8f was transiently co-expressed with and HA-βC1 or its mutant HA-βC1V32A in N. benthamiana leaves. At 60 hr post agroinfiltration (hpi), leaf lysates were immunoprecipitated with anti-GFP beads and then the precipitants were assessed by immunoblotting (IB) using anti-HA (upper panel) or anti-GFP antibodies (middle panel). (D) BiFC analyses in N. benthamiana. Representative images of nYFP-βC1 or nYFP-βC1V32A BiFC co-expressed with cYFP-NbATG8f. (E) Western blot analyses of BiFC construct combinations from the same experiments as in (D). All combinations were detected with anti-GFP polyclonal antibody.

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The following figure supplements are available for figure 1:

Figure supplement 1. N terminus of βC1 is responsible for binding to NbATG8f.
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Figure supplement 2. βC1 co-immunoprecipitated with multiple ATG8 homologs.
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Figure supplement 3. βC1 is co-localized with NbATG8f.
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Autophagy functions as an antiviral defense mechanism against CLCuMuV

Since autophagy is induced by CLCuMuV infection and βC1 specifically interacts with NbATG8f, we investigated the role of autophagy in CLCuMuV infection. For this purpose, we silenced autophagy-related genes in *N. benthamiana* using Tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) (Liu et al., 2002). Because there is functional redundancy of ATG8 genes due to the presence of multiple homologs in plants, we silenced NbATG5 and NbATG7 in *N. benthamiana*. Compared to non-silenced control plants, the mRNA levels of NbATG5 and NbATG7 were significantly reduced by gene-specific VIGS (Figure 3—figure supplement 1A), whereas we observed no obvious differences in TRV RNA levels between ATG5-silenced plants, ATG7-silenced plants, and control plants (Figure 3—figure supplement 1B). In addition, autophagy was blocked in ATG5- and ATG7-silenced plants (Figure 3—figure supplement 2A,B). It is worth noting that CLCuMuV infection had no effect on TRV-mediated VIGS (Figure 3—figure supplement 3). Furthermore, ATG5- and ATG7-silenced plants did not show any abnormal developmental phenotypes. We then infected ATG5- and ATG7-silenced plants with CA+β. We observed that the leaf curl symptoms caused by viral infection were much more severe and appeared 3 days earlier (Figure 3A,B), and CLCuMuV DNA levels were significantly higher in ATG5- and ATG7-silenced plants compared to control plants (Figure 3C). By contrast, silencing of GFP in a *N. benthamiana* GFP transgenic line 16C had no effect on CA+β infection (Figure 3—figure supplement 4).

Next, we examined the effect of enhanced autophagy on CLCuMuV infection. Consistent with the observation that down-regulating cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPCs) gene expression significantly activates autophagy (Han et al., 2015), we found that VIGS of GAPCs delayed symptom development in plants infected by CA+β (Figure 3D,E) and reduced viral DNA accumulation (Figure 3F). These results suggest that autophagy functions as an antiviral mechanism against CLCuMuV.

The βC1-NbATG8f protein interaction is involved in antiviral defense against CLCuMuV infection

To further explore the biological significance of the βC1-NbATG8f interaction on autophagy-mediated defense against CLCuMuV infection, we generated a CLCuMuB mutant (βV32A) by replacing βC1 with its mutant counterpart.
βC1V32A and inoculating this mutant virus (CA+βV32A) onto N. benthamiana leaves. Interrupting the interaction between βC1 and ATG8 accelerated the occurrence of viral symptoms and resulted in much more severe leaf curling symptoms than that caused by CA+β (Figure 4A,B). Leaf curling symptoms caused by CA+βV32A appeared 3 days earlier than the symptoms caused by CA+β (Figure 4A–B). Moreover, we observed increased viral DNA accumulation in plants infected by CA+βV32A versus CA+β (Figure 4C). Since the V32A point mutation eliminates the interaction of βC1 with NbATG8f, these results suggest that the interaction of βC1 with NbATG8 is essential for the antiviral defense mechanism of autophagy against CLCuMuV infection.

βC1 is targeted by autophagy for its degradation

Since we observed the vacuolar localization of the βC1-NbATG8f interaction and co-localization of βC1 with NbATG8f, we guess that βC1 is delivered to the vacuoles by autophagy for its degradation. To test this hypothesis, we investigated the effect of autophagy on the subcellular localization of βC1 by expressing YFP-βC1 or its mutant YFP-βC1V32A in the non-silenced control, ATG5- and

Figure 2. CLCuMuV infection activates autophagy. (A) Representative confocal microscopy images of dynamic autophagic activity revealed by specific autophagy marker CFP-NbATG8f in plants infected with CLCuMuV plus CLCuMuB (CA+β). (B) Quantification of the CFP-NbATG8f-labeled autophagic puncta per cell from (A). More than 500 mesophyll cells for each treatment were used for the quantification. Relative autophagic activity in virus infected plants was normalized to that of control plants, which was set to 1.0. Values represent means ± SE from three independent experiments. (⁎) p<0.05. (C) Representative TEM images of autophagic structures. Ultrastructure of autophagic bodies (arrows) was observed in the vacuoles of mesophyll cells of uninfected control and plants infected with CA+β. V for vacuole. (D) Autophagosome-like structures from (C) were quantified. At least 30 cells for each treatment were used for the quantification. Relative autophagic activity in virus infected plants was normalized to that of control plants, which was set to 1.0. Values represent means ± SE from three independent experiments. (⁎) p<0.05.

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The following figure supplements are available for figure 2:

Figure supplement 1. Transcription pattern of autophagy-related genes were altered during CLCuMuV infection.

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Figure supplement 2. Viral infection decreased NbJoka2/NBR1 protein level.

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Figure 3. CLCuMuV DNA accumulation is affected by host cell autophagy. (A) Viral symptoms in ATG5- and ATG7–silenced plants infected with CLCuMuV plus CLCuMuB (CA+β) at 12 dpi. Bar represents 7 cm. (B) The incidence of viral symptom appearance at different time points of post infection in ATG5- and ATG7-silenced plants. Symptom was indicated as the appearance of curled leaf caused by CA+β. Values represent means ± SE from three independent experiments. (C) Relative viral DNA accumulation in ATG5- and ATG7-silenced plants infected with CA+β. Real-time PCR analysis of V1 gene from CLCuMuV was used to determine viral DNA level. Values represent means ± SE from three independent experiments. (*) p<0.05. (D) Viral symptoms in GAPCs-silenced plants infected with CA+β at 15 dpi. Bar represents 7 cm. (E) The incidence of symptom appearance at different time points of post infection in GAPCs-silenced plants. Symptom was indicated as the appearance of curled leaf caused by CLCuMuV infection. Values represent means ± SE from three independent experiments. (F) Relative viral DNA accumulation in GAPCs-silenced plants. Real-time PCR analysis of V1 gene from CLCuMuV was used to determine viral DNA level. Values represent means ± SE from three independent experiments. (*) p<0.05.

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The following figure supplements are available for figure 3:

**Figure supplement 1.** TRV viral titers were not changed during VIGS.
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**Figure supplement 2.** Involvement of ATG5 and ATG7 in autophagy is confirmed by VIGS.
DOI: 10.7554/eLife.23897.014

**Figure supplement 3.** CLCuMuV infection has no effect on TRV-based VIGS of NbPDS.
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**Figure supplement 4.** Silencing of a non-autophagy related gene GFP has no effect on CLCuMuV infection.
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ATG7-silenced plants. As expected, we observed YFP-βC1 in the vacuoles in the non-silenced control plants. However, YFP-βC1 accumulated mostly in cytoplasm in ATG5- and ATG7-silenced plants (Figure 5A). Similarly, YFP-βC1V32A also accumulated mostly in cytoplasm in all plants, regardless of whether ATG5 / ATG7 was or not silenced (Figure 5—figure supplement 1). In addition, silencing of either ATG5 or ATG7 resulted in more accumulation of YFP-βC1 but not YFP-βC1V32A (Figure 5B and Figure 5—figure supplement 1).
We also tested the impact of autophagy on HA-βC1. Silencing of either ATG5 or ATG7 increased the accumulation of βC1 but did not affect the level of βC1V32A mutant protein or GFP alone (in the control; Figure 5C). However, no difference in the RNA level of the βC1 construct was detected in NbATG5- or NbATG7-silenced plants (Figure 5—figure supplement 2).

These results strongly suggest that βC1 is targeted by autophagy for the degradation, and the interaction of βC1 with NbATG8 is required for the autophagic degradation of βC1.
Autophagy functions as an antiviral mechanism against other geminiviruses

We then investigated the effects of autophagy on Tomato yellow leaf curl virus (TYLCV) and Tomato yellow leaf curl China virus (TYLCCNV). Silencing of ATG5 and ATG7 caused more severe disease symptoms and enhanced viral DNA accumulation compared to the control, and silencing of GAPCs reduced the severity of viral disease symptoms and viral DNA levels in plants infected by TYLCV or TYLCCNV (Figure 6). These results suggest that autophagy may have evolved as a general antiviral mechanism against various geminiviruses.

Joka2-mediated selective autophagy is not involved in antiviral defense against CLCuMuV infection

Joka2/NBR1 is the sole known selective autophagy cargo receptor in plants. To investigate the potential role of selective autophagy in antiviral defense against CLCuMuV infection, we silenced Joka2 in N. benthamiana using TRV-based VIGS (Figure 7A). Joka2 mRNA levels were significantly reduced in Joka2-silenced plants compared to non-silenced control plants (Figure 7B). However, silencing of Joka2 had no effect on CLCuMuV infection of plants with CA+β (Figure 7C). These results suggest that Joka2-mediated selective autophagy is not involved in antiviral defense against CLCuMuV infection.

Discussion

Autophagy is known to play an important role in disease resistance or susceptibility to various pathogens in plants (Han et al., 2011). However, how autophagy is linked to plant immunity remains unknown. In this study, we show that geminivirus CLCuMuV infection activates autophagy and that autophagy targets the virulence protein βC1 for degradation. Furthermore, we demonstrated for the first time that autophagy plays an active role as an antiviral mechanism in compatible plant-virus interactions.

Autophagy acts as a defense mechanism against some invading intracellular pathogens in mammalian systems (Boyle and Randow, 2013; Randow and Youle, 2014; Paul and Münz, 2016). Plant cells also employ autophagy to defend themselves against several pathogens (Han et al., 2011; Li et al., 2016). Autophagy positively regulates plant resistance against necrotrophic pathogens (Lai et al., 2011; Lenz et al., 2011; Kabbage et al., 2013) but negatively affects plant resistance against the biotrophic pathogen powdery mildew (Wang et al., 2011). Furthermore, silencing of Joka2, encoding a selective autophagy cargo receptor for polyubiquitinated cargoes, enhances susceptibility to Phytophthora infestans although it is not reported whether disrupting autophagy has an effect on plant response to this pathogen (Dagdas et al., 2016). However, we found that Joka2 silencing had no effect on CLCuMuV infection (Figure 7), implying that Joka2-mediated selective autophagy is not involved in antiviral defense against CLCuMuV in plants. CLCuMuB βC1 may not interfere with Joka2-mediated selective autophagy by depleting Joka2 out of ATG8 complexes because it has a non-classic ATG8-interacting motif (see below). In addition, silencing of autophagy-related genes caused cell death induced by Tobacco mosaic virus (TMV) to spread in inoculated leaves of N. benthamiana plants containing the TMV resistance gene N but had no effect on plant systemic resistance against TMV (Liu et al., 2005). However, the role of autophagy in compatible plant-virus interactions has been unclear. Here, we showed that disrupting autophagy enhanced plant susceptibility to three different DNA viruses, while activating autophagy enhanced plant resistance to viral infection (Figure 3 and Figure 6), suggesting that autophagy plays an active antiviral role in compatible plant-virus interactions.

Autophagy can either facilitate or suppress viral infection in mammalian cells (Orvedahl et al., 2010; Chiramel et al., 2013; Paul and Münz, 2016; Wang et al., 2016b). However, little is known about whether and/or how autophagy affects viral infection in plants. Plant viruses encode antiviral RNA silencing suppressors known as VSRs. Based on the data from autophagy inhibitor 3-MA treatment, the VSR protein P0 from Polerovirus is thought to trigger the autophagic degradation of AGO1, a component of the cellular RNAi-based antiviral defense machinery (Derrien et al., 2012). Similarly, the VSR protein VPg from Turnip mosaic virus is also reported to mediate the degradation of the cellular RNAi-based antiviral defense component SG53, partially via autophagy (Cheng and...
Figure 6. Autophagy regulates viral infection of TYLCV and TYLCCNV. (A) Viral symptoms in ATG5- and ATG7-silenced plants at 12 dpi. Bar represents 7 cm. (B) Viral symptoms in GAPCs-silenced (GAPCs VIGS) plants at 15 dpi. Bar represents 7 cm. (C) and (D) Relative viral DNA accumulation in ATG5- and ATG7-silenced plants. Real-time PCR analysis of V1 gene from TYLCCNV (C) or TYLCV (D) was used to determine viral DNA level in infected or control (TRV alone) plants. Values represent means ± SE from three independent experiments. (*) p<0.05. (E) and (F) Relative viral DNA accumulation in GAPCs-silenced plants. Real-time PCR analysis of V1 gene from TYLCCNV (E) or TYLCV (F) was used to determine viral DNA level in infected or control (TRV alone) plants. Values represent means ± SE from three independent experiments. (*) p<0.05. (G), (H), (I) and (J). The incidence of symptom appearance at different time points of post infection in VIGS plants. Symptom was indicated as the appearance of curled leaf caused by TYLCCNV in ATG5- and ATG7-silenced (G) and GAPCs-silenced (I) plants or TYLCV infection in ATG5- and ATG7-silenced (H) and GAPCs-silenced (J) plants. Values represent means ± SE from three independent experiments.

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Based on the data from an autophagy inhibitor treatment and silencing of an autophagy-nonspecific ATG gene Beclin 1, 2b protein from Cucumber mosaic virus is thought to be targeted for degradation by autophagy through the calmodulin-like protein rgsCaM (Nakahara et al., 2012). In addition, the in vitro application of an autophagy inhibitor wortmannin partially inhibited proteolysis of TYLCV proteins (Gorovits et al., 2016). In our study, by silencing of two ATG genes (ATG5 and ATG7), we clearly show that CLCuMuB C1 is degraded by autophagy. Further, autophagy genes are transcriptionally up-regulated by infection by some geminiviruses in plants (Ascencio-Ibañez et al., 2008; Miozzi et al., 2014). These observations indirectly suggest that autophagy may contribute to plant immunity during compatible plant-virus interactions. In this study, we showed that disrupting autophagy reduces plant resistance against three different plant viruses, whereas activating autophagy enhances this resistance. To the best of our knowledge, in this study, we provide the first direct evidence that autophagy functions as an antiviral mechanism in compatible plant-virus interactions.

CLCuMuB βC1 is predicted to contain two ATG8-interacting motifs (AIMs) (Kalvari et al., 2014). Interestingly, we found that an approximately 11-amino-acid motif (LVSTKSPSLIK) comprising residues 30 to 40 of βC1, but not the predicted AIMs, is responsible for the interaction of βC1 with NbATG8f. Furthermore, a point mutation at position 32 from valine to alanine in this motif eliminated the βC1-NbATG8 interaction. In addition, we found that βC1 interacted with at least four NbATG8 homologs. ATG8 proteins interact with some cargo receptors, leading to autophagic degradation of the cargoes (Johansen and Lamark, 2011). We found that disrupting autophagy increased the accumulation of βC1 protein but had no effect on the accumulation of βC1V32A (Figure 5), suggesting that βC1 is targeted for autophagic degradation. Furthermore, YFP-βC1 co-localized with CFP-NbAt8f-positive autophagic bodies in vacuoles of leaf mesophyll cells (Figure 1—figure supplement 3 and Video 1), suggesting that βC1 is targeted for autophagic degradation by binding to ATG8s.

In this study, disrupting autophagy reduced plant resistance against TYLCV, while activating autophagy enhanced plant resistance against this virus. Interestingly, autophagy also participates in resistance to TYLCV in whiteflies (Wang et al., 2016a). TYLCV induces protein aggregation in plants and whiteflies, and viral proteins (mostly viral coat protein) and ATG8 co-exist in these TYLCV-induced aggregates (Gorovits et al., 2016). Further, some AIMs are evolutionarily conserved among viruses and plants.
Plant and animal proteins (Kalvari et al., 2014; Dagdas et al., 2016), it is possible that autophagy uses similar mechanism to degrade TYLCV protein(s) in plants and whiteflies.

The βC1 protein from TYLCCNV β-satellite interacts with and is phosphorylated by SnRK1 (sucrose-nonfermenting1-related kinase 1), a plant ortholog of budding yeast SNF1 and mammalian AMPK (AMP-activated protein kinase) (Shen et al., 2011). AMPK promotes autophagy by directly phosphorylating different protein substrates involved in the initiation phase of autophagy (Cardaci et al., 2012). Interestingly, TYLCCNB βC1 also contains the polypeptide sequence LASTKSPALAK at residues 30–40, which is similar to the ATG8-binding motif of CLCuMuB βC1 (LVSTKSPSLIK). Moreover, a mutation in this motif affects TYLCCNV infection (bSPALAK at residues 30–40, which is similar to the ATG8-binding motif of CLCuMuB

The full-length infectious clones of CLCuMuV (GQ924756.1) and CLCuMuB (GQ906588.1) were described (Han et al., 2015). Previously, it was described (Dong et al., 2007) and pTRV2-LIC (Dong et al., 2007) were described previously. pTRV2-NbATG5 and pTRV2-NbATG7 were described (Wang et al., 2013). VIGS construct of NbGAPCs was described (Han et al., 2015). pTRV2-NbJoka2 was generated by cloning NbJoka2 cDNA fragment into pTRV2-LIC. Approximately 200 bp of mGFP5-ER were cloned into pTRV2 by specific primers.

The full-length infectious clones of CLCuMuV (GQ924756.1) and CLCuMuB (GQ906588.1) were described (Jia et al., 2016). The full-length infectious clones of TYLCCNV and TYLCV were described (Tao and Zhou, 2004; Zhang et al., 2009).

βC1 and its mutant βC1V32A were cloned into pGEX4T-1 vector to express GST-tagged fusion proteins in Escherichia coli. Full-length cDNA of NbATG8f was cloned into pET28a to express NbATG8f-6×His in E. coli.

Materials and methods

Plant materials

GFP transgenic16c line or wild type N. benthamiana plants were grown in pots placed in growth-rooms at 25°C under a 16-h-light/8-h-dark cycle.

Plasmid constructs

Vectors pTRV1 (Liu et al., 2002) and pTRV2-LIC (Dong et al., 2007) were described previously. pbTRV2-NbATG5 and pbTRV2-NbATG7 were described (Wang et al., 2013). VIGS construct of NbGAPCs was described (Han et al., 2015). pbTRV2-NbJoka2 was generated by cloning NbJoka2 cDNA fragment into pTRV2-LIC. Approximately 200 bp of mGFP5-ER were cloned into pTRV2 by specific primers.

The full-length infectious clones of CLCuMuV (GQ924756.1) and CLCuMuB (GQ906588.1) were described (Jia et al., 2016). The full-length infectious clones of TYLCCNV and TYLCV were described (Tao and Zhou, 2004; Zhang et al., 2009).

βC1 and its mutant βC1V32A were cloned into pGEX4T-1 vector to express GST-tagged fusion proteins in Escherichia coli. Full-length cDNA of NbATG8f was cloned into pET28a to express NbATG8f-6×His in E. coli.
the duplicated CaMV 35S promoter and the NOS terminator of pJG045, a pCAMBIA1300-based T-DNA vector (Du et al., 2013).

**Co immunoprecipitation (Co-IP) assay**

For co-IP assays, total proteins from N. benthamiana leaves (1 g leaf tissues for each sample) were extracted in ice-cold immunoprecipitation buffer (10% [v/v] glycerol, 25 mM Tris, pH 7.5, 150 mM NaCl, 1× protease inhibitor cocktail [Roche], and 0.15% [v/v] Nonidet P-40) as described (Wang et al., 2015). Protein extracts were incubated with GFP-Trap beads (ChromoTek) for 3 hr at 4°C. The precipitations were washed four times with ice-cold immunoprecipitation buffer at 4°C and were analyzed by immunoblot using anti-HA (Cell Signaling Technology), or anti-GFP (ChromoTek) antibodies.

**VIGS assay**

TRV-mediated VIGS assays were performed as described (Liu et al., 2005).

**Confocal microscopy and TEM**

Confocal imaging was performed as described (Han et al., 2015). The leaves were agroinfiltrated with autophagy marker CFP-NbATG8f for 60 hr expression, followed by an additional infiltration with 20 uM E-64d for 8 hr before being monitored by a Zeiss LSM 710 three-channel microscope with an excitation light of 405 nm, and the emission was captured at 454 to 581 nm. TEM observation was performed as described (Wang et al., 2013).

**GST pull-down assays**

GST pull-down assays were performed as described previously (Zhao et al., 2013). GST-βC1 and NbATG8f-6×His fusion proteins were produced in BL21 (DE3) cells (Stratagene).

**Yeast two-hybrid screen and interaction assays**

For yeast two-hybrid interaction assay, CLCuMuB βC1 was PCR amplified and cloned into yeast vector pYL302 to generate the LexA DNA binding domain (BD) containing bait vectors. NbATG8f was PCR amplified and cloned into the B42 activation domain (AD)-containing vector pSAH20b. The interaction assay and yeast two-hybrid screen was performed as described (Du et al., 2013).

**Real-time PCR analysis**

For expression analysis of NbATG genes, real-time RT-PCR was performed as described (Wang et al., 2013). eIF4a was used as the internal control.

For quantification of viral DNA loads, two DNA standard curves were generated. Single copy of target viral genome DNA was cloned into pMD19-T (TaKaRa, Japan) and was used as standard viral DNA (SVD) while the genome DNA of healthy N. benthamiana was served as standard genome DNA (SGD). Standard curves were generated from ten-fold dilutions of both SVD and SGD. Approximately 200 bp fragment of V1 gene from CLCuMuV/TYL/CLCCNV and 61 bp region of eIF4a gene were amplified by employing SYBR green based real-time PCR to produce standard curves. Viral DNA load in infectious plants was determined according to standard curves of SVD and SGD. Results were expressed as fold change of virus DNA from virus infected plant tissue.

For quantification of TRV viral loads, real-time RT-PCR was performed with TRV CP - specific primers (Zhao et al., 2013). eIF4a was used as the internal control.

**Western blotting**

For protein analysis, total proteins were extracted from N. benthamiana leaves using 2×Laemmlli buffer. After boiling for 10 min, protein extracts were separated by SDS-PAGE for immunoblot analysis with indicated antibodies as described (Wang et al., 2013).

**BiFC assay**

Citrine Yellow Fluorescent protein (YFP)-based Bimolecular Fluorescence Complementation (BiFC) assay was performed as described (Burch-Smith et al., 2007; Jia et al., 2016).
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Author contributions

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Author ORCIDs

Yakupjan Haxim, http://orcid.org/0000-0001-8559-0238
Yule Liu, http://orcid.org/0000-0002-4423-6045

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