Cell, Vol. 121, 567–577, May 20, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.03.007

Autophagy Regulates Programmed Cell Death during the Plant Innate Immune Response

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Summary

The plant innate immune response includes the hypersensitive response (HR), a form of programmed cell death (PCD). PCD must be restricted to infection sites to prevent the HR from playing a pathologic rather than protective role. Here we show that plant *BECLIN 1*, an ortholog of the yeast and mammalian autophagy gene *ATG6/VPS30/beclin 1*, functions to restrict HR PCD to infection sites. Initiation of HR PCD is normal in *BECLIN 1*-deficient plants, but remarkably, healthy uninfected tissue adjacent to HR lesions and leaves distal to the inoculated leaf undergo unrestricted PCD. In the HR PCD response, autophagy is induced in both pathogen-infected cells and distal uninfected cells; this is reduced in *BECLIN 1*-deficient plants. The restriction of HR PCD also requires orthologs of other autophagy-related genes including *PI3K/VPS34*, *ATG3*, and *ATG7*. Thus, the evolutionarily conserved autophagy pathway plays an essential role in plant innate immunity and negatively regulates PCD.

Introduction

Plants mount numerous defense responses to survive pathogen attack. One of these is the hypersensitive response (HR) cell death, a form of programmed cell death (PCD), which is characterized by the rapid death of plant cells at the site of pathogen infection (Lam, 2004). HR PCD is typically triggered upon recognition of a pathogen-encoded avirulence (Avr) protein by a cognate plant resistance (R) protein (Martin et al., 2003). In addition to *R* gene-mediated cell death, plants also induce cell death in response to general elicitors produced by pathogens (Boller, 1995). HR PCD typically precedes changes in ion fluxes, production of reactive oxygen intermediates (ROI) and nitric oxide (NO), cell wall strengthening, and activation of various defense-related genes (McDowell and Dangl, 2000). Collectively, these defense responses are thought to prevent the spread of the pathogen from infection sites into healthy adjacent tissue.

Considerable effort has been made to understand regulators of HR PCD, yet little is known about the molecular and cellular pathways involved in the restriction of HR PCD to pathogen infection sites. The interaction between *Nicotiana* plants that carry the *N* resistance gene and tobacco mosaic virus (TMV) is one of the classic model systems for studying plant innate immune responses (Marathe et al., 2002). The N protein belongs to a class of R proteins that contains a toll/interlukin-1 receptor homology domain, a nucleotide binding site, and a leucine-rich repeat (TIR-NB-LRR) (Martin et al., 2003). The N protein is hypothesized to specifically recognize the 50 kDa helicase domain (TMV-p50) of the TMV replicase protein to trigger induction of HR PCD and restriction of virus spread (Erickson et al., 1999). Recently, several laboratories including ours have successfully used virus-induced gene silencing (VIGS) in forward and reverse genetics screens to identify components of the *N* signaling pathway (Burch-Smith et al., 2004; Lu et al., 2003). To identify novel genes that affect initiation, execution, or restriction of TMV-induced HR PCD, we performed a high-throughput VIGS screen. We found that silencing of the putative plant autophagy gene, *BECLIN 1*, alters the HR PCD phenotype observed in plants undergoing a defense response to TMV infection.

Autophagy is a highly regulated process during which cytoplasmic materials are enclosed in double-membrane bound vesicles that are then targeted to the vacuole or lysosome for degradation (Levine and Klionsky, 2004; Ohsumi, 2001). Genetic analysis in yeast has identified at least 16 autophagy-related (*ATG*) genes that encode components of the autophagy machinery (Klionsky et al., 2003; Thumm et al., 1994; Tsukada and Ohsumi, 1993). Mutations in some of these *ATG* genes affect yeast sporulation and survival during nutrient starvation (Thumm et al., 1994; Tsukada and Ohsumi, 1993). Although *ATG* genes are conserved in higher eukaryotes, there have been few studies examining the role of autophagy in the whole organism. In *Drosophila*, autophagy is induced during starvation (Rusten et al., 2004; Scott et al., 2004) and steroid-activated PCD of salivary glands (Lee and Baehrecke, 2001). In *C. elegans*, autophagy is involved in dauer development and life span extension (Melendez et al., 2003). In mammals, autophagy regulates survival during neonatal starvation, embryonic development, and tumorogenesis (Kuma et al., 2004; Liang et al., 1999; Qu et al., 2003; Yue et al., 2003).

Little is known about the molecular functions of *ATG* orthologs in plants. Recently, it has been shown that mutations in *Arabidopsis ATG4* genes block formation of autophagic bodies and cause developmental arrest of root growth under nutrient starvation (Yoshimoto et

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al., 2004). Mutations in *Arabidopsis ATG7*, *ATG9*, and *VTI12* result in accelerated leaf senescence even under nutrient-rich conditions (Doelling et al., 2002; Hanaoka et al., 2002; Surpin et al., 2003). However, the roles of *ATG* genes and autophagy in other plant processes have not been examined.

Here we report that plant *BECLIN 1* is an ortholog of the yeast and mammalian autophagy gene *ATG6/VPS30/beclin 1*. We find that autophagy is induced during *N*-mediated defense against TMV and that *BECLIN 1* is required for this induction. Plants deficient in the autophagy genes *BECLIN 1*, *PI3K/VPS34*, *ATG3*, and *ATG7* exhibit unrestricted HR PCD in response to pathogen infection. These findings suggest that evolutionarily conserved autophagy genes and the autophagy pathway play an important role in the regulation of PCD induced during plant innate immune responses.

Results

Identification of Plant *BECLIN 1*

To identify novel genes that affect initiation, execution, or restriction of *N*-mediated HR PCD, we performed a high-throughput VIGS screen using *N* gene-containing *Nicotiana benthamiana* plants (referred to as *NN*, Liu et al., 2002b) (see Experimental Procedures for details). From 1500 cDNAs that were silenced, we found that suppression of the cDNA clone 92 (cDNA92) resulted in an altered HR PCD phenotype in response to TMV infection (see below). Sequence analysis of cDNA92 revealed significant homology to human *beclin 1* (*Hsbeclin 1)* and yeast *ATG6/VPS30* (*ScATG6/VPS30*) (Kametaka et al., 1998; Liang et al., 1999). We used RT-PCR to clone the full-length *N. benthamiana BECLIN 1* (*NbBECLIN 1*), which encodes a predicted 527 amino acid coiled-coil protein that shares 69% identity and 79% similarity with *Arabidopsis BECLIN 1* (*AtBECLIN 1*; GenBank accession NM_116036), 36% identity and 52% similarity with *Hsbeclin 1* (Liang et al., 1999), and 24% identity and 44% similarity with *ScATG6/VPS30* (Figure 1A; Kametaka et al., 1998).

Plant *BECLIN 1* Functions in Autophagy and Senescence

To test if plant *BECLIN 1* can function in autophagy, we assayed its ability to rescue autophagy-defective *ATG6/VPS30*-deficient (Δ*atg6/vps30*) yeast. Δ*atg6/vps30* yeast transformed with plant *BECLIN 1* were able to induce autophagy after nitrogen starvation in a manner similar to wild-type yeast and Δ*atg6/vps30* yeast transformed with *Hsbeclin 1* or *ScATG6/VPS30* (Figure 1B). Thus, plant *BECLIN 1* is an ortholog of yeast *ATG6/VPS30* and can functionally complement autophagy in Δ*atg6/vps30* yeast.

One known function of the *Arabidopsis* autophagy genes *ATG7* and *ATG9* and the autophagy-related gene *VTI12* is delaying leaf senescence (Doelling et al., 2002; Hanaoka et al., 2002; Surpin et al., 2003). Therefore, we examined the function of *BECLIN 1* in leaves artificially induced to senesce. The detached leaves from the *BECLIN 1*-silenced plants show accelerated senescence compared to detached leaves from control plants (Figure 1C). In addition, expression of the senescence-associated marker gene *SEN1* (AB026439) is significantly induced in *BECLIN 1*-silenced plants (data not shown). Thus, plant *BECLIN 1* is required to prevent premature senescence. This result, together with the complementation of the yeast autophagy mutant, is consistent with a role for plant *BECLIN 1* in autophagy.

BECLIN 1 Is Required to Limit TMV-Induced Cell Death to the Infection Site

Since we had identified plant *BECLIN 1* from its effect on HR PCD, we wanted to determine if the levels of BECLIN 1 RNA/protein level changed during this resistance response. For this, we examined the *BECLIN 1* levels during TMV-induced HR PCD in *NN* plants. RT-PCR and immunoblot analyses indicate that *BECLIN 1* mRNA and protein levels increase rapidly in leaves in the early stages of TMV-induced HR cell death (Figure 2A).

To elucidate the role of *BECLIN 1* in TMV-induced HR cell death, we infected *BECLIN 1*-silenced and nonsilenced *NN* plants with GFP-tagged TMV (TMV-GFP). The TMV-GFP allows us to easily follow the spread of the virus over the course of infections and hence determine how resistance response has been affected. *BECLIN 1*-silenced plants did not display any gross phenotypic abnormalities before infection with TMV-GFP (data not shown). In nonsilenced *NN* control plants infected with TMV-GFP, HR cell death was restricted to infection sites as expected (Figure 2B). In contrast, in *BECLIN 1*-silenced *NN* plants infected with TMV-GFP, HR cell death lesions were larger than those in control plants (Figure 2B). In these plants, HR lesions were visible and fully developed by 2 days postinfection (dpi), compared to 3–4 dpi in nonsilenced plants. Interestingly, in *BECLIN 1*-silenced *NN* plants, cell death extended beyond the site of infection into the uninfected tissues (Figure 2B). This unrestricted cell death resulted in the death of the TMV-GFP-inoculated leaf by 12 dpi (Figure 2B). Pathogen infection is necessary to induce cell death in *BECLIN 1*-silenced *NN* plants since mock infection of these plants fails to initiate HR cell death (Figure 2B). In the TMV-infected *BECLIN 1*-silenced *NN* plants, unrestricted cell death was not limited to the TMV-inoculated leaf since some of the upper uninoculated leaves also died (Figure 2C). These results suggest that *BECLIN 1* is required to restrict cell death to the infection site in plants undergoing HR PCD induced upon pathogen infection.

Expression of TMV-p50 Alone Is Sufficient to Cause Spreading Cell Death in *BECLIN 1*-Silenced *NN* Plants

Although the boundary of cell death expanded beyond the initial infection sites in *BECLIN 1*-silenced plants, TMV-GFP remained confined to these initial sites of infection, suggesting that the death of the uninfected tissues was independent of virus movement (Figure 2B). Moreover, death of the upper uninoculated leaves in *BECLIN 1*-silenced plant was not due to the spread of TMV-GFP from the inoculated leaf since no TMV-GFP was detected in the healthy tissues of upper uninoculated leaves either by UV illumination (Figure 2C) or by RT-PCR (Figure 2D).

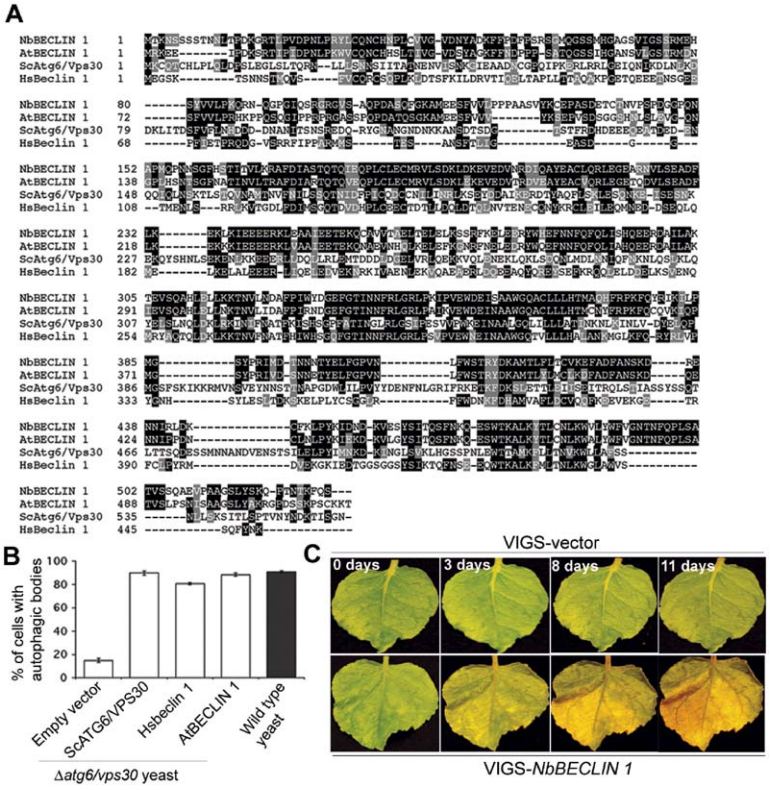

Figure 1. Identification of Plant *BECLIN 1* and Its Role in Yeast Autophagy and Plant Senescence

(A) Alignment of the predicted *N. benthamiana* BECLIN 1 (NbBECLIN 1; AY701316) amino acid sequence with *Arabidopsis* BECLIN 1 (AtBECLIN 1), yeast Atg6/Vps30 (ScAtg6/Vps30), and human Beclin 1 (HsBeclin 1). The alignment was generated using ClustalW. Numbers at left indicate amino acid residue positions. Identical residues are shaded in black, and similar residues are in gray.

(B) Plant *BECLIN 1* complements yeast autophagy mutants deficient in *ATG6/VPS30.* Cells with autophagic bodies within the vacuole were counted in nitrogen-starved wild-type yeast and in *ATG6/Vps30*-deficient yeast transformed with empty vector or with plasmids that encode *ScATG6/VPS30*, *Hsbeclin 1*, or *AtBECLIN 1*. Results shown represent the mean (±SEM) percentage of cells with autophagic bodies for triplicate samples. Results were reproduced in three independent experiments.

(C) *BECLIN 1*-deficient leaves exhibit accelerated senescence. Leaves of nonsilenced control and *BECLIN 1*-silenced plants were detached, floated on water at room temperature in the dark, and photographed after 0, 3, 8, and 11 days. Results were reproduced in four independent silencing experiments using three or more plants in each experiment.

To confirm that the cell death occurring throughout the plant in *BECLIN 1*-silenced *NN* plants is not due to the movement of the virus, we transiently expressed the *N* gene elicitor protein, TMV-p50, using an *Agrobacterium*-mediated transient expression method (Agro-infiltration). With this method, the protein of interest (in this case TMV-p50) is expressed only in the infiltrated area, as *Agrobacterium* cannot move into surrounding tissue. As expected, the expression of TMV-p50 induced cell death only in the Agro-infiltrated area of the nonsilenced *NN* control plants (Figure 3A, left panels). However, in *BECLIN 1*-silenced *NN* plants, cell death initially induced in the Agro-infiltrated area spread beyond the area of infiltration, killing the entire leaf by 13 days (Figure 3A, right panels) and eventually the uninfiltrated upper leaves (Figure 3B, right panels). Therefore, HR PCD initiated by TMV-p50 protein alone is sufficient to induce spreading cell death in *BECLIN 1*-silenced *NN* plants.

Since plant *BECLIN 1* is required for limiting genetically programmed HR PCD, we examined whether it is also required for the restriction of nonspecific cell death. We induced nonspecific cell death by treating plants with methanol. In *BECLIN 1*-silenced and nonsilenced plants, nonspecific cell death occurred at similar rates in the methanol-infiltrated area and the cell death was restricted to the infiltrated tissue (Figure 3C). These results suggest that during the *N*-mediated response, *BECLIN 1* is required to limit the spread of cell death from pathogen-infected cells into adjacent uninfected tissues. However, general "poisoning" death (i.e., non-PCD) cannot direct such signal(s) from the cell death sites to cause spread of death to the adjacent tissue even in the absence of *BECLIN 1*.

Autophagy Is Induced during *N*-Mediated Resistance to TMV

Since *BECLIN 1* is required for limiting HR PCD, we speculated that the loss of PCD restriction was due to the loss of autophagy. We therefore looked for autophagic activity in *BECLIN 1*-silenced and nonsilenced control plants before and after infection with TMV-GFP. We used LysoTracker Red dye as a probe to indicate the formation of autolysosome-like structures. This dye has been widely used as a probe to indicate autophagic activity in live tissues of various organisms because it labels acidic organelles such as autolysosomes (Moriyasu et al., 2003; Munafo and Colombo, 2001; Rusten et al., 2004; Scott et al., 2004). Before infection with TMV, cells from nonsilenced control and *BECLIN 1*-silenced plants do not incorporate LysoTracker dye (Figures 4A and 4B). However, after infection with TMV, we observed strong punctate LysoTracker-stained autolysosomes in the cells of nonsilenced *NN* control plants (Figures 4A and 4C). In contrast, these structures were rarely observed in the cells of *BECLIN 1*-silenced *NN* plants (Figures 4B and 4C). Thus, LysoTracker-stained autolysosomes are induced at the site of TMV infection during HR PCD, and *BECLIN 1* is required for this induction.

We next examined the uninfected cells surrounding the tissue undergoing HR PCD for autophagic activity. Unexpectedly, in nonsilenced *NN* control plants, a number of LysoTracker-stained autolysosomes were ob-

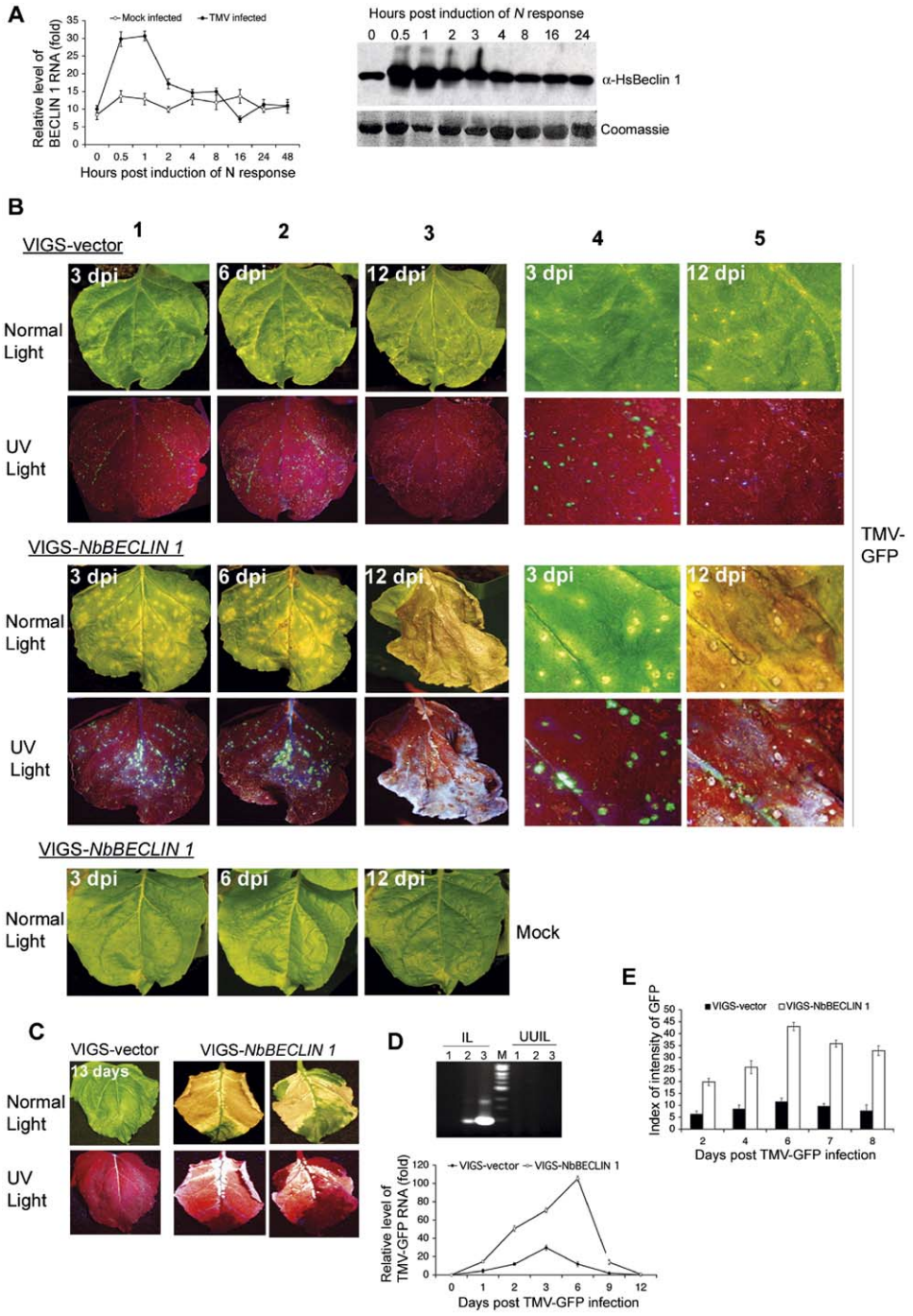

Figure 2. *BECLIN 1* Is Required to Limit the Spread of TMV-Induced PCD in *NN* Plants

(A) Expression pattern of *BECLIN 1* in plants undergoing HR response to TMV infection. Real-time RT-PCR analysis using total RNA isolated from the infected leaves and *BECLIN 1*-specific primers at time points indicated (left). Immunoblot analysis of total protein isolated from infected leaf using anti-HsBeclin 1 antibodies; Coomassie-stained membrane confirmed equal loading of samples (right).

(B) TMV-GFP-induced PCD was assessed in nonsilenced control and *BECLIN 1*-silenced plants. Mock-inoculated *BECLIN 1*-silenced plants also served as a control. Representative photographs of TMV-GFP or mock-inoculated leaves were taken under normal light and UV light. Photographs shown in panels 4 and 5 are higher magnification images of the leaves shown in panels 1 and 3, respectively. Red color in the background of GFP fluorescence in the UV light photographs is due to the autofluorescence from chlorophyll. Infection foci in 12 dpi leaves are white under UV light because virus is already cleared in these samples. Results were reproduced in at least five independent experiments using three or more plants in each experiment. dpi, days post infection.

(C) TMV-induced PCD spreads into healthy tissues of upper uninfected leaves of *BECLIN 1*-silenced plants in the absence of movement of TMV-GFP. Representative photographs from two different plants were taken under normal light and UV light.

(D) Relative levels of TMV-GFP RNA in inoculated leaf (IL) and upper uninoculated leaves (UUIL) of nonsilenced control and *BECLIN 1*-silenced plants. Ethidium bromide-stained gel represents RT-PCR product from 6 dpi samples. Lane 1, no RT control; lane 2, nonsilenced control plants; lane 3, *BECLIN 1*-silenced plants. Shown below the agarose gel is a time course qRT-PCR analysis of TMV-GFP RNA accumulation in the inoculated leaves. Error bars represent standard deviations.

(E) Quantitative analysis of GFP fluorescence in infected sites of nonsilenced control and *BECLIN 1*-silenced plants. Error bars represent standard deviations.

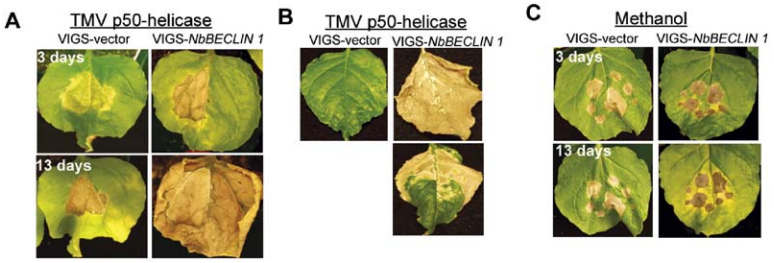

Figure 3. Expression of TMV-p50 Protein Is Sufficient to Cause a Spreading Cell Death Phenotype in *BECLIN 1*-Silenced *NN* Plants

(A) Expression of TMV-p50 using Agro-infiltration in the nonsilenced control (left) and *BECLIN 1*-silenced (right) plants.

(B) Spread of HR PCD into upper systemic leaves of *BECLIN 1*-silenced plants in which TMV-p50 is expressed in the lower leaves using Agro-infiltration.

(C) Treatment of methanol in the nonsilenced control (left) and *BECLIN 1*-silenced (right) plants.

Results were reproduced in four independent experiments using three or more plants in each experiment.

served in these cells (Figure 4D). Additionally, we also examined upper uninfected leaves of nonsilenced *NN* plants and also found a number of LysoTracker-stained autolysosomes (Figure 4E). In contrast, in *BECLIN 1*-silenced *NN* plants, very few cells surrounding the infection site and in systemic tissue contained LysoTracker-stained autolysosomes (Figures 4D and 4E).

To confirm that the LysoTracker-stained structures represent autolysosomes, we used transmission electron microscopy (TEM). In infected nonsilenced *NN* plants,

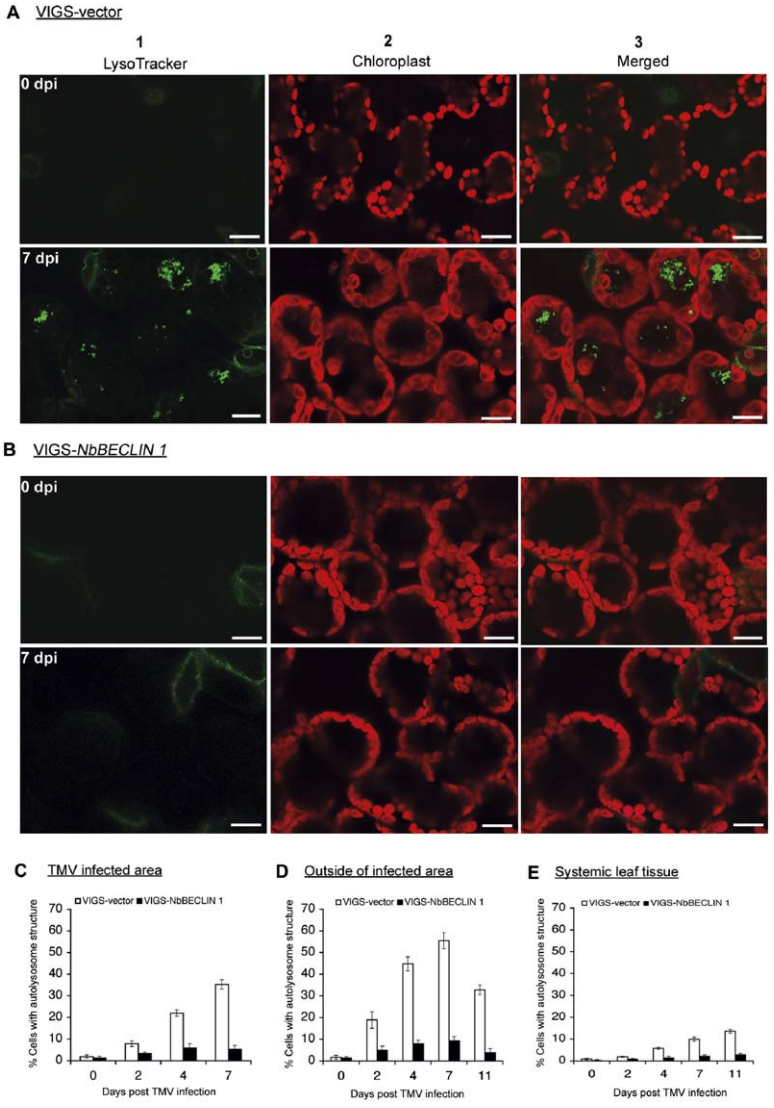

Figure 4. Autophagy Is Induced during the Resistance Response to TMV

(A and B) Representative images of LysoTracker Red-stained TMV-infected tissue derived from nonsilenced control (A) and *BECLIN 1*-silenced (B) plants. LysoTracker Red-stained punctate autolysosome-like structures inside the vacuole (green, column 1), autofluorescence from chloroplast (red, column 2), and merged images (column 3). Results were reproduced in three independent experiments using three or more plants in each experiment. Scale bar equals 50 μm.

(C–E) Quantitation of autolysosome-like structures observed in LysoTracker Red-stained infected tissue (C), uninfected tissue adjacent to infected tissue (D), and tissue from systemic leaves (E). Approximately 200–300 cells for each treatment and each time point were counted. Error bars represent standard deviations.

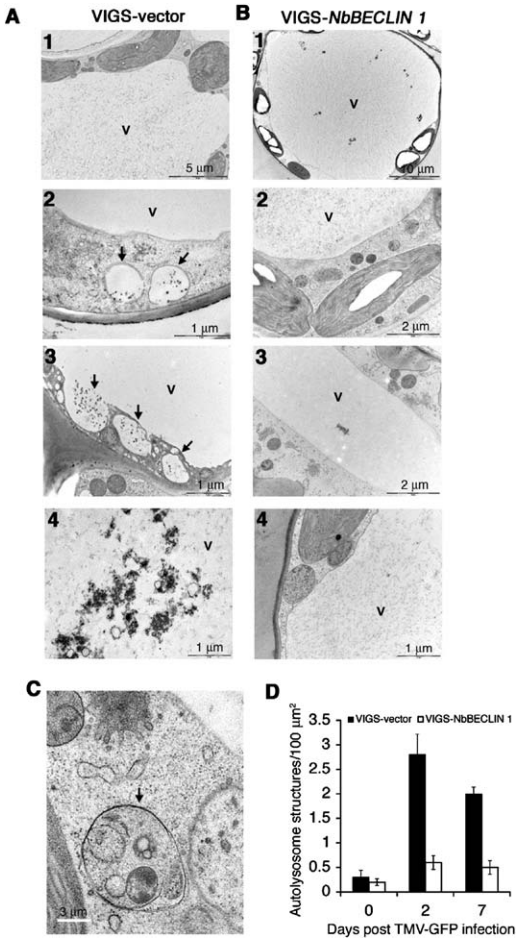

Figure 5. Transmission Electron Microscopy Images of Autophagic Bodies

(A and B) Representative transmission electron microscopy (TEM) images of healthy tissue surrounding infected tissue derived from nonsilenced control plants (A) before infection (1), 2 dpi (2), 7 dpi (3 and 4); and from *BECLIN 1*-silenced plants (B) before infection (1), 2 dpi (2), and 7 dpi (3 and 4). V, vacuole; dpi, days post infection; arrows indicate autolysosome-like structures. Results were reproduced in two independent experiments using three or more plants in each experiment.

(C) Representative TEM image of autophagosome shown at higher magnification.

(D) Quantitation of autolysosome/autophagosome structures observed in tissue surrounding infected tissue derived from nonsilenced control and *BECLIN 1*-silenced plants. Error bars represent standard deviations.

we observed a number of autolysosomes containing electron-dense material in the cytoplasm and vacuole of uninfected tissue surrounding sites of HR PCD (Figures 5A, panels 2–4, and 5D). In a few samples, we observed fusion of autolysosome structures with the vacuole (Figure 5A, panel 3). In addition, we occasionally observed double-membrane vesicles containing cytoplasmic materials and organelles; in other words, stereotypical autophagosomes (Figure 5C). In *BECLIN 1*-silenced *NN* plants, we observed reduced numbers of autolysosomes in the cytoplasm and vacuole (Figures 5B, panels 2–4, and 5D). These TEM data, together with our LysoTracker observations, indicate that autophagy is induced during the *N*-mediated defense response to TMV, both in the infection site and in uninfected adjacent tissue or systemic tissue.

BECLIN 1 Is Required to Limit HR Cell Death Induced by Other *R* Genes and General Elicitors

Since plant *BECLIN 1* is needed to contain *N*-mediated HR PCD to the infection site, we hypothesized that *BECLIN 1* might play a general role in the containment of HR PCD induced by other *R* genes as well as general elicitors. We tested the role of *BECLIN 1* in regulating HR PCD after coexpression of the bacterial resistance gene *Pto* and its cognate *Avr* gene, *AvrPto* (Pedley and Martin, 2003) and also after coexpression of the fungal resistance gene *Cf9* and its cognate *Avr* gene, *Avr9* (Hammond-Kosack and Jones, 1997). We also tested whether cell death responses to general pathogen elicitors are affected by loss of *BECLIN 1* by expressing *INF1* from *Phytophthora infestans* and by infecting plants with the bacteria *P. syringae pv. tomato* DC3000. As expected, HR cell death induced by these treatments in the nonsilenced control plants is confined to the infiltrated area even after 11 days (Figure 6). However, in *BECLIN 1*-silenced plants, cell death spread beyond the infiltrated area, killing the entire leaf by 11 days (Figure 6). In addition, death was observed in systemic upper uninfected leaves of these plants (data not shown). Together, these results demonstrate that *BECLIN 1* is not only required to limit the spread of *R* gene-mediated HR PCD triggered by viruses but also that triggered by bacteria and fungi and general elicitors.

Autophagy Genes *PI3K/VPS34*, *ATG3*, and *ATG7* Are Required to Limit Cell Death to the Infection Site

In yeast and mammals, the Atg6/Vps30/Beclin 1 protein physically associates with class III phosphoinositide 3-kinase (PI3K)/Vps34, which is also required for autophagy (Eskelinen et al., 2002; Kihara et al., 2001a; Kihara et al., 2001b). Two proteins of the conjugation pathways required for autophagy are Atg7 and Atg3 (Ichimura et al., 2000). We therefore tested the involvement of *PI3K/VPS34*, *ATG3*, and *ATG7* in TMV-induced HR PCD. Silencing these genes in *NN* plants produced a similar phenotype to that observed in *BECLIN 1*-silenced plants: although TMV-GFP was confined to the infection site, cell death was not limited to the TMV infection site and uninfected healthy tissues eventually died (Figure 7). Thus, multiple different autophagy genes function to limit the extent of spread of TMV-induced HR PCD.

Enhanced Virus Accumulation in *ATG*-Deficient Plants

Our observation of infection sites in *ATG*-silenced plants indicate that, independently of the spreading cell death phenotype, the *ATG* genes play a role in controlling TMV replication and/or cell-to-cell movement. In *BECLIN 1*-, *PI3K/VPS34*-, *ATG3*-, and *ATG7*-deficient plants, there is increased accumulation of TMV as observed by GFP fluorescence at the site of infection (Figures 2B, 2E, and 7). This increased accumulation of TMV is not due to increased spread because GFP fluo-

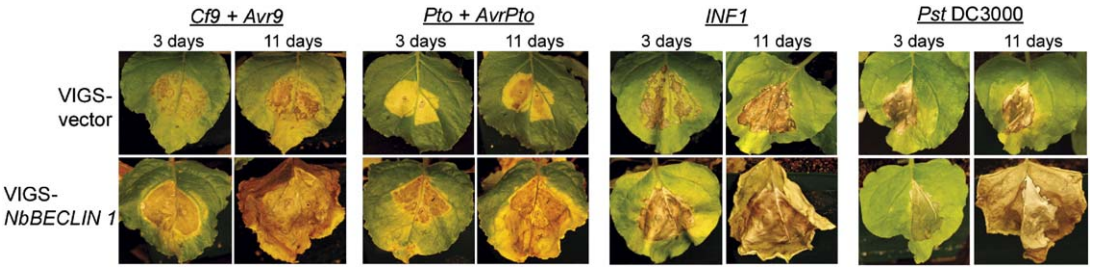

Figure 6. *BECLIN 1* Is Required to Limit Spread of HR PCD Induced during *R* Gene- and Non-*R* Gene-Mediated Resistance

Transient expression of *Cf9* with *Avr9*, *Pto* with *AvrPto*, and *INF1* using Agro-infiltration; and inoculation of *Pseudomonas syringae pv. tomato* DC3000 in nonsilenced control and *NbBECLIN 1*-silenced plants. In all cases, experiments were performed at least three times using three or more plants in each experiment.

rescence is confined only to the infection site in these plants. Furthermore, we also observed increased accumulation of TMV RNA in *BECLIN 1*-silenced plants as detected by RT-PCR (Figure 2D). This is not due to the increased spread of the virus because TMV-GFP is not detected using RT-PCR analysis in systemic uninfected upper leaves in *BECLIN 1*-silenced plants (Figure 2D). Together these results suggest that *BECLIN 1*, *PI3K/VPS34, ATG3*, and *ATG7* function not only to limit the spread of HR cell death, but also independently, to limit virus replication and/or movement.

Discussion

Role of Plant *BECLIN 1* in Autophagy and Development

We have identified plant *BECLIN 1*, an ortholog of the yeast *ATG6/VPS30* and mammalian *beclin 1* (Kametaka et al., 1998; Liang et al., 1999). In yeast, *Dictyostelium*, *C. elegans*, and mammals, *ATG6/beclin 1* is required for autophagosome formation during autophagy (Eskelinen et al., 2002; Kihara et al., 2001a; Kihara et al., 2001b; Melendez et al., 2003; Otto et al., 2003). Our results also provide evidence for plant *BECLIN 1* in autophagy. First, expression of plant *BECLIN 1* restores autophagy in autophagy-defective *ATG6/VPS34*-deficient yeast. Second, *BECLIN 1*-deficient plants exhibit accelerated leaf senescence similar to *Arabidopsis ATG4, ATG7*, and *ATG9* mutants (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004). Third, *BECLIN 1* is required for induction of autophagy during defense responses.

In mammals, *beclin 1* knockout mutant mice die early in embryogenesis, indicating that it is an essential gene (Qu et al., 2003; Yue et al., 2003). In contrast, *BECLIN 1*-silenced plants exhibited very weak developmental phenotypes, if any, under normal growth conditions. We cannot rule out the possibility that *BECLIN 1* may also have a role in early developmental processes because the VIGS screen was performed using adult plants. Furthermore, VIGS, like other RNAi techniques, fails to completely suppress expression of a target gene. Residual target gene activity may be sufficient to allow normal development to occur. However, *Arabidopsis ATG7* and *ATG9* knockout plants had a normal life cycle except that rosette leaves of these plants senesced earlier (Doelling et al., 2002; Hanaoka et al., 2002).

Plant *BECLIN 1* and Other *ATG* Genes Function as Negative Regulators of HR PCD

Generally, HR PCD initiated at the pathogen infection site is restricted to a small number of cells (Lam, 2004). In *BECLIN 1*-silenced *N* resistance gene-containing plants, HR PCD initiated at the site of TMV infection spreads into surrounding healthy tissue and into systemic uninfected leaves. Even though more TMV accu-

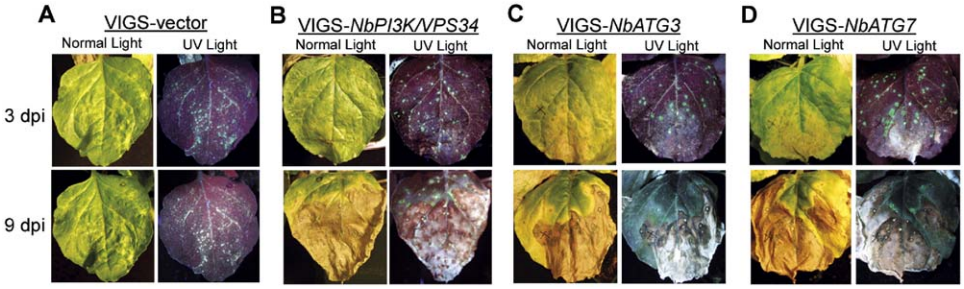

Figure 7. *PI3K/VPS34*, *ATG3*, and *ATG7* Are Required to Limit Spread of HR PCD Induced during *N*-Mediated Resistance

TMV-GFP-induced PCD was assessed in nonsilenced control (A), *PI3K/VPS34*-silenced (B), *NbATG3*-silenced (C), and *NbATG7*-silenced (D) *NN* plants. Representative photographs of TMV-GFP inoculated leaves were taken under normal light and UV light 3 dpi (top) and 9 dpi (bottom). In all cases, results were reproduced in five independent experiments using three or more plants in each experiment.

mulates in *BECLIN 1*-silenced plants at the infection site, virus was not detected outside of the infection site. In fact, local expression of *N* gene elicitor TMV-p50 protein is sufficient to induce spreading cell death phenotype in the absence of *BECLIN 1*. Therefore, movement of virus from infection site into healthy tissue is not the cause for the spreading cell death phenotype observed in these plants. This suggests that signal(s) required for inducing spreading cell death in *BECLIN 1*-silenced plants is generated after the recognition of Avr protein but not by the extent of increased pathogen replication and/or spread.

The role of *BECLIN 1* in limiting HR PCD is not restricted to the *N*-TMV system. We also observed spreading cell death during the HR in bacterial and fungal *R* gene-mediated resistance as well as general elicitor-mediated resistance in *BECLIN 1*-silenced plants. In addition to *BECLIN 1*, other *ATG* genes *PI3K/VPS30*, *ATG3*, and *ATG7* are required to restrict HR PCD to the TMV infection site. Thus, *BECLIN 1* and other *ATG* genes function in a conserved pathway to restrict HR PCD to the infection site.

If a plant is to survive an infection, HR PCD must be carefully controlled so that it does not spread throughout the plant and kill it. It has been speculated that signals generated at the site of infection are responsible for regulating HR PCD and triggering defense responses in uninfected tissues (Lam, 2004). Since plant cells are highly connected, an important long-standing question in plant innate immunity is how HR PCD is limited to the site of infection. Our data demonstrate that initiation of HR PCD is not affected in plants silenced for *BECLIN 1* or other *ATG* genes, suggesting that signals required for the induction of HR PCD are not compromised in these plants. However, the machinery required to confine PCD to the infection site is lost in these plants. Therefore, our findings provide the first genetic evidence that *ATG* genes can function in vivo as a negative regulator of HR PCD. These results contrast with findings from mammalian studies in which *ATG* genes are required to promote PCD in cells lacking intact apoptotic machinery. The autophagy genes *beclin 1* and *atg7* are required to induce nonapoptotic cell death in the murine fibroblast L929 cells treated with capsase inhibitor zVAD (Yu et al., 2004). In addition, *atg5* and *beclin 1* are required for etoposide and staurosporine treatment-induced cell death in apoptosis-resistant Bax/Bak double knockout mouse embryonic fibroblasts (Shimizu et al., 2004). However, the ability to induce apoptotic cell death in response to either UV light or serum deprivation is not compromised in ES cells derived from *beclin 1* null mutant mice (Yue et al., 2003). Moreover, recent evidence suggests that *ATG* genes promote survival during nutrient starvation or growth factor deprivation (reviewed in Levine, 2005), and RNAi of *ATG* genes triggers apoptosis in starved mammalian cells (Boya et al., 2005).

Prosurvival Function of Autophagy

Our LysoTracker staining and TEM data indicate that autophagy is induced at the site of TMV infection in *N* resistance gene-containing plants. Surprisingly, the induction of autophagy was not limited to the TMV infection sites but extended into uninfected adjacent tissue and into the systemic leaves. This was surprising because most of the evidence indicates that autophagy is induced in cells undergoing PCD, not in healthy cells. We were able to observe not only double-membrane vesicles containing cytoplasmic materials and organelles, which are representative of autophagosomes, but also autolysosome vesicles fusing with and discharging their contents into vacuoles. In contrast, *BECLIN 1*-silenced plants fail to form autolysosomes/autophagosomes and hence fail to undergo autophagy. Therefore, autophagy induced during defense responses may be required to eliminate "prodeath" signal(s) moving out of the pathogen-infected area into adjacent tissues. This could explain the increased autophagic activity observed in uninfected tissue adjacent to the infection site in control plants. It is also possible that autophagy could be involved in cleanup processes to remove cellular damage caused by proteases, hydrolases, and nucleases that are activated during the defense response. In addition, autophagy might be involved in protecting healthy cells from damage caused by ROI produced during the defense response. Thus, autophagy may play an essential "prosurvival" function in plants that effectively contains pathogen spread without resulting in "unwanted" death in innocent uninfected bystander cells.

Role of Autophagy in Antimicrobial Defense

The results presented in this study also suggest an antiviral role for autophagy in plants. In *BECLIN 1-, PI3K/VPS34-, ATG3-,* or *ATG7*-silenced plants, we observed increased accumulation of TMV at infection sites. This observation is consistent with several recent reports in mammalian cells describing a role for autophagy in host defense against viral and intracellular bacterial pathogens. Herpes simplex virus (HSV) infection triggers PKR-dependent induction of autophagy in mammalian cells, which is blocked by the HSV encoded neurovirulence factor ICP34.5 (Talloczy et al., 2002). Overexpression of mammalian *beclin 1* protects mice from fatal Sindbis virus infection (Liang et al., 1998). The mammalian autophagy gene, *atg5*, is required to degrade invasive group A *Streptococcus* (Nakagawa et al., 2004), and the induction of autophagy by starvation or rapamycin treatment promotes the degradation of *Mycobacterium tuberculosis* within phagolyososmes (Gutierrez et al., 2004). In addition, a bacterial gene product required for blocking autophagy, *iscB*, is necessary for the virulence of the intracellular enteric pathogen, *Shigella* (Ogawa et al., 2004). Our findings with TMV infection in plants suggest that the role of autophagy genes in limiting the growth of intracellular pathogens is evolutionarily conserved.

In summary, our results demonstrate that autophagy plays an important role in regulating the plant innate immune response. In the absence of *ATG* genes, the HR PCD spreads beyond the sites of infection to adjacent tissue and distal uninfected leaves. These results imply that there is a prodeath signal(s) moving out of the pathogen-infected area into adjacent tissues and distal sites that is negatively regulated by autophagy. It is not yet known whether autophagy alters the pro-

duction of death-promoting signals, prevents the movement of death-promoting signals into uninfected tissues, or protects uninfected tissues against death induced by these signals. Further genetic studies are required to determine the precise mechanistic function of autophagy in controlling PCD during plant-pathogen interactions. In addition, it will be interesting to see whether autophagy protects against PCD in other organisms and in other biological settings.

Experimental Procedures

Plant Materials, Pathogens, and Plasmids

Wild-type *N. benthamiana*, *NN* transgenic plants, and TMV-GFP were described (Liu et al., 2002b). B. Staskawicz provided *P. syringae pv. tomato* DC3000. Vectors pTRV1, pTRV2, and pTRV2-GATEWAY were described (Liu et al., 2002a; Liu et al., 2002b). pTRV2-*NtPI3K/VPS34*, pTRV2-*NbATG3,* and pTRV2-*NbATG7* were made by cloning PCR products of cDNA fragments of *PI3K/VPS34* (AY701317, bases 131–834), *ATG3* (AY701318, bases 1–493), and *ATG7* (AY701319, bases 1–558) into pTRV2. Primer sequences are available upon request. Constructs 35S:*Cf9* and 35S:*Avr9,* 35S:*Pto* and 35S:*AvrPto*, and 35S:*INF1* were provided by P. De Wit, G. Martin, and S. Kamoun, respectively.

Yeast Autophagy Complementation Assays and Artificial Induction of Leaf Senescence

Yeast autophagy complementation assays were performed as described (Liang et al., 1999). For leaf senescence assays, leaves from 2 weeks after silencing of *BECLIN 1* were detached and floated on deionized water at room temperature in the dark. Leaves of the same age were used from nonsilenced control plants in these assays. The leaves were photographed at 0, 3, 8, and 11 days after dark incubation.

Normalized cDNA Library Construction

A normalized cDNA library was constructed in pTRV2-GATEWAY vector by custom services from Invitrogen (CA) using poly(A)$^+$ RNA isolated from a 1:1 mixture of tissue derived from TMV-resistant *NN* plants and TMV-susceptible *N. benthamiana* plants at 0, 1, 4, 8, 16, and 24 hr after TMV infection.

VIGS, Pathogen-Induced Cell Death Assay, and TMV-GFP Quantification

NN plants were used for TMV-GFP and TMV-p50 helicase assays; *N. benthamiana* plants lacking the *N* gene were used for other assays. For the VIGS screen, *Agrobacterium* cultures (OD_{600} = ~0.8) containing pTRV1 and individual clone from pTRV2-normalized cDNA library were mixed at a 1:1 ratio and infiltrated into the lower leaves of 4-leaf-stage plants using a 1 ml needleless syringe. We screened a total of 1500 cDNA clones containing *Agrobacterium* colonies. To monitor the effect of silencing a given gene on the defense response, 10 days after infiltration the upper leaves of the plants were infected with TMV-GFP. These plants were monitored for changes in HR cell death and loss of resistance to TMV by assaying movement of TMV-GFP into the upper uninoculated leaves. GFP imaging was performed using UV illumination, and photographs were taken using an Olympus Camedia E10 digital camera. TMV at the infection site was quantified by measuring pixel size of the GFP spot using Adobe Photoshop. At least 20–30 infection sites from four independent infected leaves were used for quantification of GFP intensity for each treatment.

VIGS of *BECLIN 1*, *PI3K/VPS34*, *ATG3*, and *ATG7* was performed as described (Liu et al., 2002b). We used 10 days postsilenced plants for TMV infections since RT-PCR analysis of silenced plants showed significant reduction of *BECLIN 1* RNA at this time point compared to nonsilenced plants (data not shown). Silenced plants and nonsilenced plants were infected with TMV-GFP or were infiltrated with *Agrobacterium* carrying TMV-GFP T-DNA or 35S:TMV-p50 or 35S:*Cf9* and 35S:*Avr9* or 35S:*Pto* and 35S:*AvrPto* or 35S:*INF1*. For experiments in which *Cf9* and *Avr9* or *Pto* and *AvrPto* were coexpressed, *Agrobacterium* cultures carrying these plasmids were mixed in 1:1 ratio while maintaining an OD_{600} = 0.8. *Pst* DC3000 were resuspended in 10 mM $MgCl_2$ to OD_{600} = 0.4 and infiltrated into leaves.

RT-PCR and Quantification of mRNA Levels

Detection of TMV-GFP by RT-PCR was performed using the protocol described (Liu et al., 2002b). *BECLIN 1* and TMV RNA levels were monitored by SYBR Real-Time RT-PCR using RNA extracted from tissue collected at 0, 0.5, 1, 2, 4, 8, 16, 24, and 48 hr in which the *N* response was synchronously induced (Liu et al., 2004). Detection of real-time RT-PCR products was done by the incorporation of the fluorescent DNA dye SYBR Green using the QuantiTect SYBR Green PCR kit (Qiagen) following the manufacturer's recommendations in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, USA). All calculations and statistical analysis were performed as described in the User Bulletin.

Immunoblot Analysis

To detect *BECLIN 1* protein levels, the *N* response was induced synchronously (Liu et al., 2004) and tissue was collected at 0, 0.5, 1, 2, 3, 4, 8, 16, and 24 hr. Protein extraction and immunoblot analysis using HsBeclin 1 antibody (provided by Atsuki Nara) was performed as described (Liu et al., 2004).

LysoTracker Red Staining and Transmission Electron Microscopy

BECLIN 1-silenced or nonsilenced control plants were infiltrated with *Agrobacterium* carrying TMV-GFP. At 0, 2, 4, 7, and 11 days post TMV-GFP infiltration, the infected area, areas adjacent to the infected area, and upper uninfected leaves were infiltrated with 100 μM E-64d. Plants were kept at room temperature overnight and the E-64d infiltrated parts of the leaves were excised and immediately vacuum infiltrated with 1 μM LysoTracker Red DND-99 (Molecular Probes, Eugene, OR) and kept in the dark for 1 hr. Leaf pieces were then placed with the adaxial surface down into a single-well Lab-Tek II chambered 1.5 cover glass system submerged in 1 μM LysoTracker Red. Confocal images were acquired on a Zeiss Axiovert 200M equipped with a Zeiss LSM 510 NLO laser scanning microscope using a 40X C-Apochromat (NA 1.2) water-corrected objective lens. All data were collected with 543 nm excitation of a 1 mW helium:neon laser with 565–625 nm band pass and 650 nm long pass emission filters for LysoTracker Red and chloroplast autofluorescence, respectively. For quantification of LysoTracker-stained structures, approximately 200–300 cells for each treatment and each time point were counted.

Electron microscopy was performed using a modified protocol described from Bourett et al. (1999). Briefly, leaf discs were quickly and carefully excised from E-64d or 1 μM concanamycin A infiltrated regions with a 1.5 mm diameter biopsy punch, transferred to a dextran-filled 1.5 mm by 200 mm HPF flat specimen carrier, and then frozen using a Leica EMPACT high-pressure freezer. Samples were subsequently freeze-substituted in a Leica EM AFS at –90°C for 3 days in 4% OsO_4 in acetone, warmed slowly to room temperature, rinsed three times in acetone, and embedded in Embed-812 resin. Sectioned samples were stained with lead citrate and uranyl acetate before imaging on a Zeiss CEM 902 transmission electron microscope equipped with a Megaview II digital camera (Soft Imaging System, Boulder, CO). To quantify autophagic structures, digital images were acquired and the number of autolysosomes or autophagosomes was determined per 2500 mm^2 field.

Acknowledgments

We thank T. Burch-Smith, M. Seay, and J. Soosaar for helpful comments on the manuscript; B. Staskawicz, G. Martin, P. De Wit, and S. Kamoun for providing reagents; and A. Nara for HsBeclin 1 antibody. Supported by NSF Plant Genome grant DBI-0116076 and NIH grant GM62625 to S.P.D.-K. and NIH grant AI151367 to B.L.

Received: December 21, 2004
Revised: February 1, 2005
Accepted: March 7, 2005
Published: May 19, 2005

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