CpATG8, a Homolog of Yeast Autophagy Protein ATG8, Is Required for Pathogenesis and Hypovirus Accumulation in the Chest Blight Fungus

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Autophagy is a degradation system in the cell, involved in the turnover of cellular components, development, differentiation, immune responses, protection against pathogens, and cell death. Autophagy is induced by nutrient starvation, in which cytoplasmic components and organelles are digested via vacuoles/lysosomes. In this study, by using electron microscopy, we observed that hypovirus CHV1-EP713 infection of Cryphonectria parasitica, the causative agent of chestnut blight disease, caused proliferation of autophagic-like vesicles. This phenomenon could be mimicked by treating the wild-type strain of the fungus EP155 with the autophagy induction drug rapamycin. Some of the hypovirulence-associated traits, including reduced pigmentation and conidiation, were also observed in the rapamycin-treated EP155. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) revealed that genes involved in autophagy were up-regulated in expression. Deletion of cpatg8, a gene encoding a homolog of ATG8 in Saccharomyces cerevisiae, resulted in attenuation of virulence and reduction in sporulation, as well as accumulation of the double-stranded viral RNA. Furthermore, virus-encoded p29 protein was found to co-localize with CpATG8, implying that the viral protein may interfere with the function of CpATG8. Taken together, these findings show that cpatg8 can be regulated by the hypovirus and is required for virulence and development of the fungus and accumulation of viral dsRNA in chestnut blight fungus.

Keywords: cpatg8, autophagy, hypovirus, virulence, chestnut blight fungus

INTRODUCTION

Autophagy is a conserved cellular process of eukaryotic cells that degrades intracellular protein complexes and organelles in the vacuole or lysosome (Klionsky et al., 2016; Liu et al., 2016; Yin et al., 2016). Autophagy has diverse physiological functions in the regulation of energy and nutrient metabolism, organelle quality control, removing misfolded proteins (Yin et al., 2016), and the development of filamentous fungi (Khan et al., 2012; Voigt and Pöggeler, 2013b; Liu et al., 2016). Among many molecular elements, ATG8, a ubiquitin-like protein, is a key element of autophagy pathway (Klionsky et al., 2016). In Saccharomyces cerevisiae, it was reported that ATG8...
is conjugated to the lipid phosphatidyl ethanolamine (PE) and required for autophagosome formation (Nakatogawa et al., 2007). ATG8 is localized to preautophagosomal structures (PAS), autophagosomes, and autophagic bodies (Suzuki et al., 2001). In filamentous fungi, autophagy has been shown to be involved in virulence, cellular growth, development, and environmental stress (Pollack et al., 2009; Bartoszewska and Kiel, 2011; Klionsky et al., 2016; Liu et al., 2016). In Fusarium graminearum, FgATG8 was found to function in the formation of aeriel mycelium and formation of reproductive structures, nutritional use of storage lipid droplets, and infection (Josefsen et al., 2012).

Autophagy could be induced by various abiotic and biotic stresses including pathogen infection (Hayward and Dinesh-Kumar, 2011). In addition, the canonical function of autophagy may play a role in antiviral activities (Dagdas et al., 2016; Clavel et al., 2017; Hafren et al., 2017; Haxim et al., 2017). However, different animal and plant viruses have developed diversified strategies to evade or hijack the autophagy pathway to promote their own infection or transmission (Dong and Levine, 2013; Chen et al., 2017). Although the role of autophagy in host-virus interactions in animals and plants has been studied to some extent, functions of autophagy in the fungus–virus interface remain to be understood.

Chestnut blight caused by Cryphonectria parasitica is a well-known forest disease. Infection with hypoviruses, a group of plus sense RNA viruses, attenuates virulence of this fungus (Hayward and Nuss, 2005; Eusebio-Cope et al., 2005). In this report, we observed that both hypovirus-induced drug rapamycin in the wild-type strain EP155 could cause proliferation of autophagic-like vesicles, and expression of their own infection or transmission (Josefsen et al., 2015; Hafrén et al., 2017; Haxim et al., 2017; Lan et al., 2008; Choi et al., 2012). In addition, the canonical function of autophagy may play a role in antivirus activities (Dagdas et al., 2016; Clavel et al., 2017; Hafren et al., 2017; Haxim et al., 2017). However, different animal and plant viruses have developed diversified strategies to evade or hijack the autophagy pathway to promote their own infection or transmission (Dong and Levine, 2013; Chen et al., 2017). Although the role of autophagy in host-virus interactions in animals and plants has been studied to some extent, functions of autophagy in the fungus–virus interface remain to be understood.

**TABLE 1** | List of primers used.

| Primer name | Sequence 5’-3’ | Name of gene |
|-------------|----------------|--------------|
| Hyg-F       | CTGAAATACAGGAAATAAATG | hph |
| Hyg-R       | AGAACCGACATCATCATGAGG | cpatg8 |
| cpatg8-all-F | CGTGGTGAGTACTTTTGAGTGA | cpatg8 |
| cpatg8-all-R | CTGGCCACTCAGGTACCTGGTA | cpatg8 |
| cpatg8-LF | TACTCTTTTCGCTGCTGCTTGGG | cpatg8 |
| cpatg8-LR | ATATGATCTCTTGGACACTGGCA | cpatg8 |
| cpatg8-RF | GTTTTCTCAGGAGTCTGCTCGTGG | cpatg8 |
| cpatg8-RR | CAGAAATCTGAGTCCGAGCTGACATG | cpatg8 |
| Δcpatg8-com-F | CGGAAATCTGACGGTCTGCTGGG | cpatg8 |
| Δcpatg8-com-R | CTAGGCGGCGCGATGCTGCTGCTG | cpatg8 |
| 18S-F | TCTCGAATCTGGAGCGGCCGC | 18S rRNA |
| 18S-R | TATCGTGTTGATACCGCGG | 18S rRNA |
| cpatg1-F | TCCACAAACTCTGTCATGCTGAGG | cpatg1 |
| cpatg1-R | TGTTGCGACACAGCTATGAGT | cpatg1 |
| cpatg3-F | GGGTCTGCGTGGACCTTTCGCA | cpatg3 |
| cpatg3-R | ATGAACTTCAAGCCACACAGC | cpatg3 |
| cpatg4-F | GCGTGCAACAGAAGCTGAGA | cpatg4 |
| cpatg4-R | GTATCGGTTGATGAGGAAAT | cpatg4 |
| cpatg7-F | GCGTGCAACAGAAGCGAATA | cpatg7 |
| cpatg7-R | AGAGAGAAGAGGCGGCTTCCCTC | cpatg7 |
| cpatg8-F | ATCCAGTCAAGGAGTACGC | cpatg8 |
| cpatg8-R | ATCCAGTCAAGGAGTACGC | cpatg8 |
| cpatg18-F | CTGCGACCGGCGTGACCTGCTGG | cpatg18 |
| cpatg18-R | AGCGCGTCTCGCTCGCTGGT | cpatg18 |
| cpatg33-F | ACAAGCTTCCGCCCAAGAGCAGG | cpatg33 |
| cpatg33-R | TGCTTCTGAGGAGTCTGCTG | cpatg33 |
| p29-F | ATAGGCGGCGCGATGCTGCTGCTG | p29 |
| p29-R | ATAGTTAACCAATCCGGGCAAGGG | p29 |

**Gene Manipulation**

Gene cloning, PCR, and Southern analysis were performed according to Sambrook and Russell (2001). Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and listed in Table 1.

**Construction and Complementation of cpatg8 Null Mutants**

cpatg8 null mutants were constructed by homologous recombination. Briefly, a fragment containing a hygromycin-resistant gene hph in place of the cpatg8 coding region that was flanked with cpatg8 sequences was generated by PCR. This fragment was introduced into DK80 spheroplasts via the PEG-mediated transformation protocol. Putative cpatg8 disruptants were screened by PCR, selected for nuclear homogeneity by single-spore isolation, and further verified by Southern blot analysis. Confirmed transformants were designated as Δcpatg8 strains. A 2.55 kb genomic fragment with EcoRI and NotI containing the complete cpatg8 transcript region...
FIGURE 1 | Hypovirus infection and rapamycin treatment resulted in the accumulation of autophagosome-like vesicles. (A) Transmission electron micrographs of the hyphae. The hypha morphology of EP155 and EP155/CHV1-EP713 did not show apparent distinction. However, the intracellular structure of EP155/CHV1-EP713 showed much more membrane structures. Arrow indicates autophagosome-like vesicle. (B) The quantification of the average number of autophagosome-like vesicles per cell and a minimum of 20 cells were counted. Rapamycin-treated EM graphs (14 days on rapamycin-supplemented PDA plate). ** indicates \( P < 0.001 \), determined by Student's \( t \)-test.

FIGURE 2 | Sporulation level and growth rates of hypovirus infection and rapamycin treatment on \( C. \) parasitica. (A) Phenotypes on PDA. Photo was taken at day 7. (B) Sporulation level. Spores were counted on day 14. (C) Growth rates of EP155, EP155/CHV1-EP713, and EP155 (rapamycin treatment, 60 nM). Growth rate was measured from cultures grown on PDA for 7 days. Values are means ± S.E.M of three independent experiments. ** indicates \( P < 0.01 \) and *** indicates \( P < 0.001 \), determined by Student's \( t \)-test.

FIGURE 3 | Transcript accumulation levels of autophagy-related genes. The transcript accumulation level for each of the target genes in EP155 was set at 1.0, and the corresponding levels in EP155/CHV1-EP713 were expressed as a percentage of that of EP155. Values were calculated from three biological repeats. Bars indicate mean deviations.

Construction of GFP-Labeled CpATG8 and RFP-Labeled p29 Strains

GFP-CpATG8 fusion plasmid (pCPXG418-GFP-cpatg8) was constructed as described (Shi et al., 2014). CpATG8 encoding region sequence was amplified and cloned into the pCPXG418-GFP to construct pCPXG418-GFP-cpatg8. Likewise, the virus-encoded protein p29 encoding region sequence was amplified by PCR with primer pair p29-F/R (Table 1) and cloned into the pCPXHY2-RFP to generate the recombinant plasmids pCPXHY2-RFP-p29. Subsequently, these two recombinant plasmids were transformed into the protoplasts of the wild-type

(0.69 kb), promoter region (1.20 kb), and terminator region (0.66 kb) was amplified by PCR and then inserted into the transformation vector pCPXG418 to generate the construct pCPXG418-cpatg8. Complemented strains were constructed by transforming \( \Delta \)cpatg8 spheroplasts with pCPXG418-cpatg8 and the complemented transformants were validated by PCR and Southern blot.
strain EP155, respectively. The expression of GFP and RFP was observed using an Olympus BX51 fluorescence microscope.

**Electron Microscopy**

For scanning electron microscopy, fungal samples (2 × 4 mm) were prepared after 5 days of cultivation on PDA. Samples were fixed in 2% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 6.8) at 4°C for 4–6 h and then washed with the same buffer for 2 h. The samples were dehydrated in a graded acetone series (30, 50, 70, 80, 90, and 100%) with each grade kept for 30 min and three times in 100% acetone. Finally, the fully dehydrated samples were dried in a Critical Point Dryer (HCP-2, Hitachi), mounted on stubs, and then coated with gold about 200 nm in thickness in a Sputter Coater (S-3400N, Hitachi). The coated specimens were observed with a SEM HV (S-3400N, Hitachi) at 10 kV.

For transmission electron microscopy, hyphae cultivated on PDA medium for 7 days were scraped with a clean scalpel and washed three times with sterilized distilled water, fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C overnight, rinsed three times with phosphate buffer (50 mM, pH 6.8), and post-fixed overnight in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.0) at 4°C for 2 h. After rinsing with phosphate buffer, the samples were dehydrated in a gradient ethanol series and embedded in Epon 812 resin. The ultrathin sections were stained in 2% uranium acetate followed by lead citrate and visualized under a transmission electron microscope (Hitachi, H-7650) operating at 80 kV.

**Quantification of Gene Transcripts and Viral dsRNA**

The relative accumulation of gene transcripts in the strain DK80 and Δcpatg8 was measured using quantitative real-time RT-PCR as previously described (Shi et al., 2014). Total cDNA was synthesized using an amount of 4 µg of RNA with appropriate gene-specific primers (Table 1). The real-time PCR was performed in a LightCycler 480 (Roche Applied Science)
and normalized against that of 18S rRNA. The viral dsRNA accumulation level was examined using the method described previously (Lin et al., 2007). RNA samples stained with ethidium bromide were scanned using a Typhoon 9410 phosphorimager (GE Healthcare Life Sciences). To quantify the relative amount of large and medium dsRNA, the scanned gel image analysis was performed by ImageQuant TL-1D gel analysis software. 18S rRNA was used as the normalization reference.

**Virulence Assays**

Virulence was tested on dormant stems of Chinese chestnut (*Castanea mollissima*) with five replicates per fungal strain as previously described (Yao et al., 2013). The inoculated stems were kept at room temperature in a plastic bag to maintain moisture for 4 weeks. After incubation for 4 weeks, canker sizes were measured and the results were subjected to statistical analysis using the PROCGLM procedure (SAS, version 8.0). The type I error rate was set at $P < 0.05$.

**RESULTS**

**Hypovirus Infection Promotes Autophagy in *C. parasitica***

Previous studies showed that CHV1-EP713 and virus-encoded protein p29 were presented in vesicles (Dodds, 1980; Jacob-Wilk et al., 2006; Wang et al., 2013). We used an electron microscope to compare the subcellular structure of the wild-type strain EP155 and virus-infected strain EP155/CHV1-EP713 and found that the number of vesicles was increased in the cytoplasm by more than 3-fold following virus infection (Figure 1).

Rapamycin can induce autophagy (Klionsky et al., 2016). EP155 treated with rapamycin resulted in a similar increase in vesicle numbers (Figure 1). Moreover, rapamycin-induced EP155 exhibited the phenotype similar to that of EP155/CHV-1EP713, including decreased sporulation, growth, and pigment production (Figure 2). To understand whether the autophagy pathway could be induced upon CHV1-EP713 infection, qRT-PCR was performed to compare transcripts of autophagy-related genes. Results showed that expression of *cpatg1*, *cpatg3*, *cpatg4*, *cpatg7*, *cpatg8*, *cpatg18*, and *cpatg33* was significantly up-regulated ($P < 0.05$) by 5.79-, 3.45-, 2.59-, 4.32-, 3.55-, 2.74-, and 3.36-fold, respectively (Figure 3). Thus, it was concluded that virus infection stimulated autophagy in *C. parasitica*.

**cpatg8 Is Essential for Autophagy in *C. parasitica***

The putative *atg8* homolog was inspected against the *C. parasitica* genome database (http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=Crypa2&id=102797). The coding region of the *cpatg8* gene is composed of three exons with 124 amino acid residues and two introns of 314 bp. The deduced
CpATG8 protein showed a high level of homology (94–97% amino acid identity) compared to those of Magnaporthe oryzae, Neurospora crassa, Pichia pastoris, and Aspergillus nidulans, with Chaetomium thermophilum and M. oryzae being the highest at 97% and A. nidulans being the lowest at 94% (Figure S1).

To investigate the functions of the cpatg8 gene, cpatg8 disruption mutants were constructed via homologous recombination with a hygromycin-B-resistant cassette (Figure 4A). Three randomly selected single-spore-derived transformants were screened by PCR and further confirmed by Southern blot analysis (Figures 4B–D). The conidiation level of the Δcpatg8 strain was drastically reduced and aerial hyphae were significantly less than those of the parent strain DK80 and wild-type strain EP155 (Figures 4E,F). As shown in Figure 4E, the day–night growth patterns of Δcpatg8 were different from its parental or the wild-type strain. The abnormal phenotype of the mutants could be fully restored following reintroduction of the wild-type cpatg8 gene (Figure 6), suggesting that cpatg8 is a gene essential for autophagy in C. parasitica.

Deletion of cpatg8 Attenuates C. parasitica Virulence and Reduces Accumulation of the Hypovirus RNA

EP155 and parental strain DK80 were highly virulent and incited large cankers on chestnut stems, whereas Δcpatg8 caused very small cankers, similar to those of EP155/CHV1-EP713. Virulence of the Δcpatg8 mutant was restored following reintroduction of the wild-type cpatg8 gene (Figure 6). cpatg8 is known to be a key gene of autophagy that functions at different stages of the autophagy pathway (Klionsky et al., 2016). To conclusively establish whether autophagy was required for replication of CHV1-EP713, Δcpatg8 was paired with hypovirus-infected strain EP155/CHV1-EP713. While the converted Δcpatg8 colonies showed virally infected phenotypes of reduced conidiation and loss of pigmentation, the accumulation level of the viral dsRNA was significantly reduced (Figure 7), demonstrating that cpatg8 plays an important role in CHV1-EP713 replication.

Viral Protein p29 Co-localizes With CpATG8

ATG8 has been used as a marker for autophagy-related structures in a wide range of eukaryotes (Pollack et al., 2009; Voigt and Pöggeler, 2013a; Yin et al., 2016). By co-expression of GFP-CpATG8 and viral p29-RFP in EP155, it was observed that these two proteins co-localized in the vesicles of the cell (Figure 8), likely in the autophagosomes.
DISCUSSION

Autophagy is an evolutionarily conserved biological process found in eukaryotic cells involved in recycling processes. Autophagy can be measured by using fluorescent marker-tagged Atg8 and be inhibited by deletion of autophagy-related genes (Veneault-Fourrey et al., 2006; Duan et al., 2013; Sumita et al., 2017; Ren et al., 2018). Consistently, loss of cpatg8 leads to the inhibited autophagy in C. parasitica (Figure 4), suggesting that cpatg8 is essential for autophagy in this fungus.

Disruption of atg8 has been reported to result in reduced conidiation, impaired aerial mycelial growth, and attenuated virulence in several pathogenic fungi. In the rice blast fungus M. oryzae, it was shown that autophagy was necessary for the blast disease (Kershaw and Talbot, 2009). Disruption of MgATG8 resulted in autophagy-arrested conidial cell death and loss of virulence (Veneault-Fourrey et al., 2006; Liu et al., 2010). In the corn smut fungus Ustilago maydis, autophagy was required for pathogenicity (Nadal and Gold, 2010). In Botrytis cinerea, BcATG8 is essential for autophagy to regulate fungal development and pathogenesis, and deletion of BcATG8 blocked autophagy and significantly impaired aerial hyphal growth, reproductive development, and virulence (Ren et al., 2018). In this regard, our current study was in accordance with the previous findings that ATG8 is required for conidiation and virulence.

Induction of autophagy and exploitation of components of autophagy pathway in favor of viral replication and spread have been reported for many RNA and DNA viruses. For example, HIV blocks the formation of mature autolysosomes in macrophages and exploits the autophagic component during early stages in replication (Kyei et al., 2009). Poliovirus uses autophagy components for genome replication, while dengue and Zika viruses use autophagy components for post-replication processes (Abernathy et al., 2019). In our study, accumulation of autophagosome-like vesicle was found in both CHV1-EP713-infected and rapamycin-treated strains (Figure 1), and genes involved in autophagy were up-regulated in hypovirus-infected strain (Figure 3), suggesting that hypovirus may induce and exploit autophagy for its genome replication. Reduced accumulation of hypoviral dsRNA in cpatg8 null mutant (Figure 7) further supports this assumption. As a matter of fact, it has been reported that hypovirus infection induces proliferation of the vesicle in C. parasitica (Dodd, 1980; Wang et al., 2013) and hypovirus dsRNA and viral encoded p29 had been found to co-fractionate with a trans-Golgi network-derived membrane (Jacob-Wilk et al., 2006), implying that the hypovirus may be trafficked by the vesicles.

The hypovirus-encoded p29 is a multifunctional protein involved in virus replication, suppression of host RNA interference system, post-transcription modification, sporulation, host symptom development, and virulence (Choi et al., 1991; Suzuki et al., 2003; Andika et al., 2019). Our studies also showed hypovirus CHV1-EP713 protein p29-RFP co-localized with GFP-CpATG8. Whether p29 is involved in the process of autophagosome fusion with lysosomes remains to be determined.

AUTHOR CONTRIBUTIONS

LS carried out the experiment and helped to drafted the manuscript. JW participated in the culture of fungal strains, data analysis, and helped to draft the manuscript. RQ and FY participated in the culture of fungal strains and data analysis. JS designed and supervised the experiment and drafted the manuscript. BC designed and supervised the experiment and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00222/full#supplementary-material

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