The autophagy protein BcAtg2 regulates growth, development and pathogenicity in the gray mold fungus *Botrytis cinerea*

Na Liu1,2, Sen Lian1,2, Baohua Li1,2 and Weichao Ren1,2*

**Abstract**

Autophagy is an intracellular degradation process that facilitates material recycling to maintain cellular homeostasis in eukaryotes. Atg2 is a phospholipid transfer protein involved in cellular autophagy in *Saccharomyces cerevisiae*. To date, the role of Atg2 in growth, development and pathogenicity of the gray mold fungus *Botrytis cinerea* remains unknown. In this study, we identified and characterized an Atg2 ortholog, designated as BcAtg2, in *B. cinerea*. Deletion of BcATG2 resulted in a block of the autophagic process in *B. cinerea*. The ΔBcAtg2 mutant failed to produce sclerotia, and showed significant reduction in mycelial growth rate, formation of aerial mycelium and conidiation. In addition, the ΔBcAtg2 mutant lost the ability to form infection structures and cause symptom on host plants. All of these phenotypic changes in ΔBcAtg2 mutant were restored by targeted gene complementation. Moreover, BcAtg2 was demonstrated to physically interact with the phosphoinositide binding protein BcAtg18. Taken together, these results indicate that BcAtg2 plays an important role in vegetative growth, development and pathogenicity in *B. cinerea*.

**Keywords:** *Botrytis cinerea*, Autophagy, BcAtg2, Growth, Development, Pathogenicity

**Background**

*Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*) is a filamentous fungal pathogen that causes gray mold on a wide range of hosts (more than 500 plant species), including fruits, vegetables and ornamental flowers (Fillinger and Elad 2016). Gray mold occurs in both pre- and post-harvest period, leading to considerable crop losses worldwide (Williamson et al. 2007). Currently, due to the lack of resistant varieties, chemical control is the most effective strategy for the control of gray mold (Dean et al. 2012), however, the control efficiency of some fungicides has been severely weakened with the development of fungicide-resistant populations in *B. cinerea* (Hu et al. 2016). Understanding the fundamental biology of this pathogen will provide the basis for establishment of more effective control strategies.

Autophagy is an evolutionarily conserved intracellular degradation process in eukaryotes, which plays an important role in the maintenance of cellular homeostasis (Yorimitsu and Klionsky 2005). The process of autophagy includes sequestration of bulky cytosolic contents such as long-lived proteins and damaged organelles into vesicular compartments, followed by delivery of these intracellular materials to lysosomes/vacuoles for degradation (Mizushima 2007). A central event in autophagy is the biogenesis of double-membrane vesicles termed autophagosomes, which sequester intracellular components for degradation in lysosomes/vacuoles (Xie and Klionsky 2007). Previous studies in yeast and mammals have shown that the autophagy-related protein Atg2 forms a complex with the phosphatidylinositol 3-phosphate (PI3P)-binding protein Atg18 to initiate autophagosome biogenesis in the preautophagosomal structure.
(PAS) (Chowdhury et al. 2018; Kotani et al. 2018). In filamentous fungi, Atg2 is essential for autophagy, and mediates fungal development and pathogenicity in Magnaporthe oryzae and Fusarium graminearum, two important plant pathogens causing destructive diseases on grassy crops (Khan et al. 2012; Lv et al. 2017).

Up to now, the role of Atg2 in B. cinerea, a model organism for molecular studies of necrotrophic fungi, has not been reported. In the present study, we identified an ortholog of yeast Atg2 in B. cinerea and investigated its biological functions. Our data demonstrated that BcAtg2 plays important roles in mycelial growth, conidiation, sclerotial formation and pathogenicity in B. cinerea.

Results
Identification of BcATG2 in B. cinerea
The BcAtg2 protein-coding gene BcATG2 (Gene ID: BCIN_14g01550) was identified through BLASTP search of the B. cinerea genome database (https://fungi.ensembl.org/Botrytis_cinerea/Info/Index) using the Saccharomyces cerevisiae Atg2 protein as a query. BcATG2 is predicted to encode a 2160-aa protein, which shares 27% identity with Atg2 of S. cerevisiae. Protein domain architectures were characterized by using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/), and the results showed that BcAtg2 contains typical functional domains of Atg2, including chorein-N, ATG-CAD and ATG-C (Fig. 1a). Phylogenetic analysis of BcAtg2 and its orthologs from different fungal species revealed that BcAtg2 is evolutionarily conserved in fungi (Fig. 1b).

To investigate the role of BcATG2 in B. cinerea, the ΔBcAtg2 mutant was generated by targeted deletion of BcATG2 using a homologous recombination strategy (Fig. 2a). The putative BcATG2 deletion mutants were preliminarily screened from the hygromycin-resistant transformants by PCR amplification (Fig. 2b). Southern blot analysis confirmed that the recombination event of BcATG2 mutant occurred at the right locus (Fig. 2c). BcAtg2 is essential for autophagy
GFP-BcAtg8 is a useful marker for monitoring autophagy in B. cinerea (Ren et al. 2018a). To determine the role of BcAtg2 in autophagy, the subcellular localization of GFP-BcAtg8 was analyzed in B. cinerea wild-type and BcATG2 deletion mutant strains. Under nitrogen-starvation conditions (nitrogen-deficient medium supplemented with phenylmethylsulfonyl fluoride, PMSF), the autophagic bodies were observed in the vacuole of the wild-type strain B05.10, whereas no autophagic bodies were observed in the vacuole of the BcATG2 deletion mutant ΔBcAtg2 (Fig. 3a). Additionally, the proteolysis...
of GFP-BcAtg8 was analyzed. Under nutrient-rich conditions, both the GFP-BcAtg8 fusion protein and free GFP protein were detected by anti-GFP western blotting in B05.10, and nitrogen starvation promoted the proteolysis of GFP-BcAtg8, however, the protein contents of GFP-BcAtg8 and GFP were not affected by nitrogen starvation in ΔBcAtg2 (Fig. 3b, c). These results indicate that BcAtg2 plays an important role in the regulation of autophagy in B. cinerea.

BcAtg2 is involved in growth, conidiation and sclerotial formation

To determine the role of BcAtg2 in growth and development of B. cinerea, we compared the characteristics of mycelial growth, conidiation and sclerotial production between the wild-type strain B05.10, BcATG2 deletion mutant ΔBcAtg2 and complemented strain BcAtg2-C. Compared with B05.10 and BcAtg2-C, ΔBcAtg2 showed significant reduction in aerial mycelium formation and mycelial growth rate after incubation on PDA, MM or CM medium at 25 °C for 3 days (Fig. 4a, b). After incubation on sterilized potato fragments at 20 °C under white light for 10 days, ΔBcAtg2 formed abnormal conidiophores (Fig. 5a), and its conidial morphology was also altered (approximately 25%) (Fig. 5b). In addition, the production of conidia in ΔBcAtg2 was drastically reduced compared with those in B05.10 and BcAtg2-C (Fig. 5c). As for sclerotial formation, B05.10 and BcAtg2-C produced abundant sclerotia, whereas ΔBcAtg2 failed to produce sclerotia after incubation on PDA at 10 °C in the dark for 3 weeks (Fig. 6). These results indicate that BcAtg2 is crucial for growth, conidiation and sclerotial formation in B. cinerea.

BcAtg2 is required for pathogenicity

To determine the role of BcAtg2 in the pathogenicity of B. cinerea, we conducted infection tests on cucumber leaves and apple fruits. At 3 days post-inoculation, the wild-type strain B05.10 and the complemented strain BcAtg2-C caused significant lesions in both cucumber
leaves and apple fruits, whereas the \( \Delta BcAtg2 \) mutant lost ability to infect both host tissues (Fig. 7a, b). Further investigation revealed that \( \Delta BcAtg2 \) lost the ability to form infection structures at the initial stage of infection (Fig. 7c). These results indicate that BcAtg2 is essential for pathogenicity in \( B. \text{cinerea} \).

**BcAtg2 physically interacts with BcAtg18**

In yeast, Atg2 forms a complex with Atg18, which is essential for autophagosome formation (Kobayashi et al. 2012). To test whether an interaction exists between their orthologs in \( B. \text{cinerea} \), yeast two-hybrid (Y2H) assays were performed. The results demonstrated that an interaction exists between BcAtg2 and BcAtg18 (Fig. 8). These results suggest that the corresponding regulatory mechanism is evolutionarily conserved in \( B. \text{cinerea} \).

**Discussion**

In the last decades, the study of autophagy has been extended from yeast to plants and mammals. Increasing evidence has demonstrated that autophagy plays a wide range of biological roles in eukaryotes by maintaining cellular homeostasis (Awan and Deng 2014; Parzych and Klionsky 2014). The role of autophagy in plant pathogenic fungi has been gradually revealed through investigation of gene functions (Pollack et al. 2009). In the present study, we identified and characterized the Atg2 ortholog in the gray mold fungus \( B. \text{cinerea} \). The results indicated that BcAtg2 is essential for autophagy, and is involved in the regulation of growth, development and pathogenicity in \( B. \text{cinerea} \).

Atg2 forms a complex with Atg18, which is essential for autophagosome formation in yeast (Yamamoto and Ohsumi 2014). In this study, the direct physical interaction between BcAtg2 and BcAtg18 was verified by...
yeast two-hybrid assay. Additionally, the autophagic process was blocked in the \textit{BcATG2} deletion mutant. Functional domain analysis showed that \textit{BcAtg2} contains chorein-N, ATG-CAD and ATG-C, the typical conserved domains in yeast \textit{Atg2} that are involved in the regulation of autophagy (Osawa et al. 2019). Phylogenetic analysis suggested that \textit{Atg2}-coding genes are conservatively evolved to maintain a similar function in fungi.

Endogenous nutrient recycling supplied by autophagy ensures normal growth and development of pathogenic fungi (Bartoszewska and Kiel 2010). Consistent with the crucial role of \textit{Atg2} in mycelial growth and conidiation in \textit{M. oryzae} and \textit{F. graminearum} (Khan et al. 2012; Lv et al. 2017), in this study, \textit{ΔBcAtg2} exhibited reduced mycelial growth rate and aerial mycelium formation, and poor conidiation. In addition, \textit{ΔBcAtg2} lost the ability to form sclerotia. These results suggest that \textit{BcAtg2}-mediated autophagy is required for pathogenicity of \textit{B. cinerea}.

Autophagy is a cellular degradation process, through which a large amount of material and energy needs are met in pathogenic fungi during their infection of host plants (Deng et al. 2012). Previous studies have shown that autophagy is involved in pathogenicity of plant pathogens (Pollack et al. 2009). In this study, \textit{ΔBcAtg2} was shown to lose the ability to form infection structure and therefore failed to infect plant tissues, consistent with the \textit{ATG2} deletion mutants of \textit{M. oryzae} and \textit{F. graminearum}, which also lose pathogenicity (Khan et al. 2012; Lv et al. 2017). These results suggest that \textit{BcAtg2}-mediated autophagy is required for pathogenicity of \textit{B. cinerea}.
Conclusions
In this study, we identified and characterized the autophagy protein Atg2 in the gray mold fungus *B. cinerea*, and our results indicate that Atg2-mediated autophagy plays important roles in the regulation of growth, development and pathogenicity in *B. cinerea*. These results provide a better understanding of the biological role of autophagy in fungi.

Methods
Strains and culture conditions
The *B. cinerea* wild-type strain B05.10 and derivative transformants were routinely cultured on potato dextrose agar (PDA), minimal medium (MM) and complete medium (CM) at 25 °C as described previously (Ren et al. 2018a). Mycelia were cultured in liquid yeast
extract peptone dextrose (YEPD) medium with shaking (180 rpm) at 25 °C for DNA/RNA extraction. Sterilized potato fragments were used for conidia production. PDA was used for sclerotial formation. MM-N [MM without (NH₄)₂SO₄], a nitrogen-deficient medium, was used for induction of autophagy.

### Targeted gene deletion and complementation

To replace BcATG2 in the wild-type strain B05.10, 1296-bp upstream and 1323-bp downstream flanking sequences of the gene were amplified by PCR using the genomic DNA of B05.10 as template. The resulting amplicons were fused with hygromycin phosphotransferase gene (HPH) through double-joint PCR (Yu et al. 2004). Protoplast preparation and transformation were performed as described previously (Gronover et al. 2001). The resulting hygromycin-resistant transformants were preliminarily screened by PCR with primers shown in Additional file 1: Table S1, and further confirmed by Southern blot analysis. For complementation assays, BcAtg2-GFP cassette was constructed according to the method described previously (Ren et al. 2018b). Briefly, the entire open reading frame (ORF) of BcATG2 (without stop codon) was amplified and cloned into pNAN-OGG vector using the One Step Cloning Kit (Vazyme, Nanjing, China). The resulting constructs were confirmed by sequencing and transformed into the BcATG2 deletion mutant.

### Yeast two-hybrid assay

To construct vectors for yeast two-hybrid tests, the full-length cDNA of BcATG2 and BcATG18 were amplified from the genomic cDNA of B05.10, and then cloned into the yeast GAL4 activation domain vector pGADT7 and GAL4 binding domain vector pGBK T7, respectively, using a One Step Cloning Kit (Vazyme, Nanjing, China). The resulting vectors were confirmed by sequencing and co-transformed into the yeast reporter strain AH109 following the standard protocol (Schiestl and Gietz 1989). The yeast transformants growing on synthetic medium (SD) lacking Leu and Trp were collected and assayed for growth on SD (5 mM 3-aminotriazole) lacking Leu, Trp and His. The interactions between pGADT7-T and pGBK T7-53, and between pGADT7-T and pGBK T7-Lam were served as the positive and negative control, respectively. The experiments were repeated three times.

### Western blot assay

Total proteins were extracted as described previously (Gu et al. 2015). Equal amounts of proteins were loaded into 10% sodium dodecyl sulfate–polyacrylamide gel. After electrophoresis, proteins were transferred onto polyvinylidene fluoride (PVDF) membrane with a Tanon electroblotting apparatus. The anti-GFP antibody (Abcam, Cambridge, Cat#Ab32146) and anti-GAPDH antibody (Hangzhou HuaAn Biotechnology Co. Ltd, Hangzhou, China, Cat#EM1101) were used for immunoblot analyses. The experiments were repeated three times.

### Pathogenicity assay

Infection tests were performed on cucumber leaves and apple fruits. Briefly, the plant samples were point-inoculated with mycelial plugs (5 mm in diameter) from 3-day-old cultures of the B. cinerea wild-type strain B05.10 and derivative strains. Before inoculation, plant cuticles were punctured with a sterilized needle tip to facilitate penetration. Water agar plugs without fungal mycelia were used as negative controls (mock). The inoculated samples were incubated under conditions of high relative humidity (about 95%) at 25 °C with 16 h of daylight. The experiments were repeated three times and each time with at least ten samples.

### Abbreviations

BLAST: Basic Local Alignment Search Tool; Co-IP: Co-immunoprecipitation; DIC: Differential interference contrast microscope; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GFP: Green fluorescent protein; PCR: Polymerase chain reaction; SMART: Smart Modular Architecture Research Tool; YEPD: Yeast extract peptone dextrose.

### Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-022-00108-2.

### Additional file 1: Table S1

Primers used in this study.

### Acknowledgements

We thank Prof. Changjun Chen (Nanjing Agricultural University) for the constructive comments on this manuscript.

### Authors’ contributions

BL and WR designed the research. NL and SL performed the experiments. NL and WR analyzed the data. NL and WR wrote the manuscript. All authors read and approved the final manuscript.

### Funding

This research was supported by the National Natural Science Foundation of China (32001937), Natural Science Foundation of Shandong Province (ZR2020QC125), and the Scientific Research Fund for High-level Talents in Qingdao Agricultural University (665/1120060 and 663/1121023).

### Availability of data and materials

Not applicable.

### Declarations

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.
Competition interests
The authors declare that they have no competing interests.

Author details
1 College of Plant Health and Medicine, Qingdao Agricultural University, Qingdao 266109, China.
2 Engineering Research Center of Fruit and Vegetable Pest Precision Control of Qingdao, Qingdao 266109, China.

Received: 15 November 2021 Accepted: 19 January 2022 Published online: 03 February 2022

References
Awan MUF, Deng Y. Role of autophagy and its significance in cellular homeostasis. Appl Microbiol Biotechnol. 2014;98:5319–28. https://doi.org/10.1007/s00253-014-5721-8.

Bartoszewska M, Kiel JA KW. The role of macroautophagy in development of filamentous fungi. Antioxid Redox Signal. 2010;14:2271–87. https://doi.org/10.1089/ars.2010.3528.

Chowdhury S, Otomo C, Leitner A, Ohashi K, Aebersold R, Lander GC, et al. Insights into autophagosome biogenesis from structural and biochemical analyses of the ATG2A-ATG4P complex. Proc Natl Acad Sci USA. 2018;115(2):5979–81. https://doi.org/10.1073/pnas.1811874115.

Dean R, van Kan JAL, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, et al. The top 10 fungal pathogens in molecular plant pathology. Mol Plant Pathol. 2012;13(4):414–30. https://doi.org/10.1111/j.1364-3703.2011.00783.x.

Deng Y, Qu Z, Naqvi NL. Role of macroautophagy in nutrient homeostasis during fungal development and pathogenesis. Cells. 2012;1(3):449–63. https://doi.org/10.3390/cells10300449.

Filling DIE, Elad Y. Botrytis-the fungus, the pathogen and its management in agricultural systems. Cham: Springer; 2016. https://doi.org/10.1007/978-3-319-23371-0.

Gronover CS, Kasulke D, Tudzynski P, Tudzynski B. The role of G protein alpha subunits in the infection process of the gray mold fungus Botrytis cinerea. Mol Plant-Microbe Interact. 2001;14(11):1293–302. https://doi.org/10.1094/MPMI.2001.14.11.1293.

Gu Q, Zhang C, Liu X, Ma Z. A transcription factor Fgs1Ste 12 is required for pathogenicity in Fusarium graminearum. Mol Plant Pathol. 2015;16(1):1–13. https://doi.org/10.1111/mpp.12155.

Hu MJ, Cox KD, Schnabel G. Resistance to increasing chemical classes of fungicides by virtue of “Selection by Association” in Botrytis cinerea. Phytopathology. 2016;106(12):1513–20. https://doi.org/10.1094/PHYTO-04-16-0161-R.

Khan IA, Hu JP, Liu XH, Rehman A, Lin FC. Multifunction of autophagy-related genes in filamentous fungi. Microbiol Res. 2012;167:339–45. https://doi.org/10.1016/j.micres.2012.01.004.

Kobayashi T, Suzuki K, Ohsumi Y. Autophagosome formation can be achieved in the absence of Atg18 by expressing engineered PAS-targeted Atg2. FEBS Lett. 2012;586(16):2473–8. https://doi.org/10.1016/j.febslet.2012.06.008.

Kotani T, Kirisako H, Koizumi M, Kishimoto Y, Nakatogawa H. The Atg2-Atg18 complex tethers pre-autophagosomal membranes to the endoplasmic reticulum for autophagosome formation. Proc Natl Acad Sci USA. 2018;115(41):10363–8. https://doi.org/10.1073/pnas.1806727115.

Lv W, Wang C, Yang N, Que Y, Talbot NJ, Wang Z. Genome-wide functional analysis reveals that autophagy is necessary for growth, sporulation, deoxynivalenol production and virulence in Fusarium graminearum. Sci Rep. 2017;7:11062. https://doi.org/10.1038/s41598-017-11640-z.

Mizushima N. Autophagy: process and function. Gene Dev. 2007;21:2861–73. https://doi.org/10.1101/gad.1599207.

Osawa T, Kotani T, Kawaoka T, Hirata E, Suzuki K, Nakatogawa H, et al. Atg2 mediates direct lipid transfer between membranes for autophagosome formation. Nat Struct Mol Biol. 2019;26:281–8. https://doi.org/10.1038/s41594-019-0203-4.

Parzych KR, Klionsky DJ. An overview of autophagy: morphology, mechanism, and regulation. Antioxid Redox Signal. 2014;20(3):460–73. https://doi.org/10.1089/ars.2013.5371.

Pollack J, Harris SD, Marten MR. Autophagy in filamentous fungi. Fungal Genet Biol. 2009;46(1):1–8. https://doi.org/10.1016/j.fgb.2008.10.010.

Ren W, Liu N, Sang C, Shi D, Zhou M, Chen C, et al. The autophagy gene BcATG8 regulates the vegetative differentiation and pathogenicity of Botrytis cinerea. Appl Environ Microbiol. 2018;84(11):e02455-e2517. https://doi.org/10.1128/AEM.02455-17.

Ren W, Sang C, Shi D, Song X, Zhou M, Chen C. Ubiquitin-like activating enzymes BcAtg3 and BcAtg7 participate in development and pathogenesis of Botrytis cinerea. Curr Genet. 2018;64:919–30. https://doi.org/10.1007/s00294-018-0810-3.

Schiestl RH, Gietz RD. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr Genet. 1989;16:339–46.

Williamson B, Tudzynski B, Tudzynski P, van Kan JAL. Botrytis cinerea: the cause of grey mould disease. Mol Plant Pathol. 2007;8(5):561–80. https://doi.org/10.1111/j.1364-3703.2007.00417.x.

Xie Z, Klionsky DJ. Autophagosome formation: core machinery and adaptations. Nat Cell Biol. 2007;9:1102–9. https://doi.org/10.1038/nclb.2007.1102.

Yamamoto H, Ohsumi Y. The molecular mechanisms underlying autophagosome formation in yeast. In: Hayat MA, editor. Autophagy: cancer, other pathologies, inflammation, immunity, infection, and aging. Cambridge: Academic Press; 2014. p. 67–77. https://doi.org/10.1016/B978-0-12-405529-2.00004-4.

Yorimitsu T, Klionsky DJ. Autophagosome: molecular machinery for self-eating. Cell Death Differ. 2003;10:1542–52. https://doi.org/10.1038/sj.cdd.4401765.

Yu JH, Hamari Z, Han KH, Seo JA, Reyes-Dominguez Y, Scaglioni P. Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet Biol. 2004;41(11):973–81. https://doi.org/10.1016/j.fgb.2004.08.001.

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