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Exocyst subunit *BcSec3* regulates growth, development and pathogenicity in *Botrytis cinerea*

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Botrytis cinerea is a saprophytic plant pathogenic fungus that can infect a variety of crops and cause gray mold, which leads to huge losses worldwide. The role of exocyst in fungal pathogenicity is being revealed. In this study, homologous recombination technology was used to knock out the exocyst subunit *BcSec3* of *B. cinerea*, and it was found that the *BcSec3* subunit plays a crucial role in the growth and pathogenicity of *B. cinerea*. Compared with the wild-type strain B05.10, the mycelial growth ability of the *BcSec3* deletion strain was reduced by up to 49.8%, the conidia production capacity of the deletion strain was severely lost, and no sclerotia was formed. The polygalacturonase, is one of plant cell wall hydrolases, whose activity in *BcSec3* deletion strain was significantly reduced. In the tomato leaves infection assay *in vitro*, the lesion area caused by the *BcSec3* deletion strain was only 20% of the wild type after 5 days of infection. Observation by light microscope showed that the morphology of *BcSec3* deletion strain mycelium was significantly changed, the mycelium became thinner and deformed, and the polarity growth was not obvious. Further observation with laser confocal microscopy and transmission electron microscopy was conducted. It was found that compared with the wild type, the number of vesicles in *BcSec3* deleted cells reduced and localization and distribution of vesicles changed. In mutant cell, vesicles relatively concentrated in the cytoplasm, while in wild-type cell mainly concentrated inside the cell membrane. These evidences indicate that the exocyst subunit *BcSec3* plays an important role in the growth, development and pathogenicity of *B. cinerea*.

Keywords. *Botrytis cinerea*; *BcSec3*; growth; pathogenicity

1. Introduction

Botrytis cinerea is a saprophytic phytopathogenic fungus that occurs worldwide and causes gray mold in more than 1400 host plants, including solanaceous vegetables, cucurbits, strawberries and ornamentals (Elad *et al*. 2016; Dean *et al*. 2012). *B. cinerea* not only affects the growth period of the plant, but also its storage (Katiyar *et al*. 2015). The economic losses caused by *B. cinerea* are huge, reaching up to 10 billion US dollars per year worldwide (Patel *et al*. 2015).

As a typical saprophytic plant pathogenic fungus, *B. cinerea* uses toxic secondary metabolites as molecular weapons to induce plant cell death and feed on dead organisms (Antoine *et al*. 2019). *B. cinerea* is difficult to control because it can exist in multiple forms such as mycelium, conidia, and sclerotia, all the three forms of *B. cinerea* are able to infect plants and can survive for a long time even under adverse conditions (Williamson *et al*. 2007). *B. cinerea* can make plants susceptible to diseases by secreting toxic factors such as oxalic acid, reactive oxygen species, hydrolase, and toxins (Petrasch *et al.* 2019). Chemical control by using fungicides is still one of the most effective and economical methods of gray mold management currently. However, *B. cinerea* has developed resistance to almost all available classes of fungicides, multi-fungicide resistant strains have been found worldwide. The heavy use

of chemicals has a negative impact on the environment and human health, and contributes to the selection of resistant pathogenic strains (Nanni *et al*. 2016).

Filamentous fungi are a morphologically diverse group of extremely polarized organisms, exhibiting continuous growth at their hyphal tips (Caballero-Lima *et al*. 2013). Fungal hyphae apical growth process involves the polarized traffic of secretory vesicles to the Spitzenkörper (SPK) and subsequent distribution to plasma membrane, where the materials for cell wall expansion are provided, it also provide some factors needed for growth (Fisher and Roberson 2016). It is found that the SPK work as a vesicle supply center (VSC) for the vesicles to accumulate. Even if there are no SPK in some fungi, there still are some similar structures perform same function (Fisher and Roberson 2016). The formation of SPK and the polarized growth of fungal cells have been demonstrated to be related to exocyst.

Exocyst plays a crucial role in the docking and tethering of post-Golgi secretory vesicles to the plasma membrane in eukaryotic cell. The exocyst was first identified in *Saccharomyces cerevisiae* and then found in filamentous fungi at growth sites (Chen *et al*. 2015). Exocyst is essential for polarized delivery of secretory vesicles and cell growth. Exocyst affects fungal morphogenesis by ensuring the transport of substances through vesicles. It was reported that vesicles carry a cargo enriched with virulence factors, thus, play an important role in pathogen-host interactions (Zhang *et al*. 2019).Exocyst is an octameric protein complex that consisting of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 in *S.cerevisiae* (TerBush and Novick 1995).At the tip of elongated cells of budding yeast, clusters of vesicles are observed, providing a reservoir of material necessary for rapid cellular extension or synaptic activity (Kim and Rose 2015).

In recent years, the exocyst had been proven important for filamentous fungi, such as *Aspergillus niger, Magnaporthe oryzae, Cryptococcus neoformans,* and *Neurospora crassa* (Kwon *et al*. 2014; Giraldo *et al*. 2013; Panepinto *et al*. 2009; Riquelme *et al*. 2014).In *A.nidulans,* the destruction of exocyst subunit leads to abnormal growth, and the deletion of Sec15 results in a decrease in the secretion of polar vesicles of *Candida albicans* (Herrero *et al*. 2014; Guo *et al*. 2016).Studies on the pathogenic factor of rice blast *M.oryzae* has shown that exocyst complexes play a special role in the pathogenesis process by regulating the secretion of effector protein subpopulations (Gupta *et al*. 2015). More and more research is revealing the crucial role of exocyst in the generation of cell polarity as well as the delivery of effector proteins in filamentous fungi and pathogenicity on plant hosts (Chen *et al*. 2015).

In fungi, the components of the exocyst complex are well conserved. The specific functions of each component of the exocyst in biology and pathogenesis are not fully understood (Chen *et al.* 2015). Sec3, one subunit of exocyst, binds to the t-SNARE protein, and functions in membrane fusion and vesicle tethering in yeast (Yue *et al*. 2017). Sec3 localizes to the exocytic site independently of other components, being a spatial landmark for polarized secretion (Bendezú *et al*. 2012).In yeast, the deletion of *Sec3* would decrease the secretion of proteins and other factors, which affects the growth (Tay *et al*. 2019).

According to *B. cinerea strain* B05.10 genomic database from Ensembl Fungi, each subunit of exocyst sequence was verified to exist, there is only one candidate copy of *BcSec3* in genome of *B. cinerea strain* B05.10 (Bcin03g04910). The putative gene is 5032 bp, contains 4 exons and 3 introns. The predicted translation product is 1468 amino acids. This study focuses on the dissection of the function of *BcSec3* in *B. cinerea* strain B05.10. In this study, we found that *BcSec3* showed its importance in regulation of growth, development and pathogenicity of *B. cinerea*.

2. Materials and methods

2.1 *Strains, media, and culture*

B. cinerea haploid strain *B05.10* was a gift from Dr. P. Tudzynski (Institut fur Botanik, Institut fur Botanik, Westfalische Wilhelms Universitat, Germany).*B. cinerea* strain *B05.10* was used as the recipient strain for gene replacement and complementation, as well as the wild-type (*WT*) strain in all experiments. *B. cinerea* was cultured on potato dextrose agar (PDA) containing 2% glucose, 20% potato extract, 2% agar, with 12 h light and 12 h dark. Potato dextrose broth (PDB) was used as liquid medium for collection of mycelia and extracellular proteins. SH (Schenk and Hildebrandt) agar medium containing 0.6 M sucrose, 5 mM HEPES, 1 mM $(NH_4)_2HPO_4$, and 1% agar was used to regenerate *B. cinerea* protoplasts.

2.2 *Construction of large homologous fragments for recombination*

B. cinerea genomic DNA was extracted with DNA Extraction Kit (BioFlux, Japan).For the replacement of

BcSec3, hygromycin phosphotransferase gene(*hph*) was used to link with upstream (FlankL) and downstream(FlankR) coding region fragments. The *hph* gene of 1895 bp containing its promoter *PgpdA* and terminator *trpC* was amplified from plasmid pAN7-1 with primer pair Hyg-F(5′-TCCAAGTCGCCTCCACGAATGAAGTAGGTAGAGCGAGTAC-3′) and Hyg-R(5′-ATGACTACCTGCACCCTCCTACGACCGTTGATCTGCTTGA-3′).The FlankL, containing a 636 bp upstream *BcSec3* coding region was amplified with PCR primer pair BFL-F(5′-GCTGCGTTTGTTCTGCTTGG-3′) and BFL-R(5′-GTACTCGCTCTACCTACTTCATTCGTGGAGGCGACTTGGA-3′). The FlankR containing a 693 bp downstream *BcSec3* coding region was amplified with PCR primer pair BFR-F(5′-TCAAGCAGATCAACGGTCGTAGGAGGGTGCAGGTAGTCAT-3′) and BFR-R(5′-TACAGGCCGTAATTCAAACG-3′). The homologous recombination knockout fragment (3224 bp) was obtained by overlapping extension PCR with three fragments FlankL, *hph* and FlankR. BFL-R and Hyg-F have a complementary pairing sequence of 20 bp, and BFR-F and Hyg-R have a complementary pairing sequence of 20 bp. Therefore, the three amplified fragments can be connected in order by overlapping extension PCR. The PCR products were all purified using kit SanPrep (SangonBiotech, Shanghai).

2.3 *Construction of BcSec3 deletion strains and complementation*

The preparation of protoplasts is same as described by Guan (Guan *et al.* 2020). Protocols for transformation were slightly modified from an established procedure (Zhang *et al*. 2014). 200 µL suspension of 4×10^7 cells were pre-cooled on ice for 5 min. Then 150 µg of transformation fragments dissolved in 100 µl of KC buffer (containing 2.5 mM spermidine) was added to the protoplasts. The mixture was incubated for 5 min on ice. Then 200 µl of 25% PEG 3350 (Sigma) in 50 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.5) was added gently which was then kept on ice for 20 min. 400 µl of the same PEG solution was added, and incubated for 20 min at room temperature. The mixture was added into SH agar, after an incubation at 22°C for 24 h, the regenerating protoplasts were overlaid with 10 mL of PDA containing 50 µg of hygromycin B per mL. After incubating for 3 to 5 days at 22°C, the emerging colonies were transferred to PDA plates containing 70 µg of hygromycin B. To verify the insertion site of the *Hph* gene in the transformants, the forward primer Sec3-F 5′-GCTGCGTTTGTTCTGCTTGG-3 ′ and the reverse primer Hph-R 5′-TACAGGCCGTAATTCAAACG-3 ′were used for PCR to confirm whether the target gene was replaced by *Hph* gene.

The *BcSec3* deletion mutant was complemented with the full *BcSec3* genomic sequence of 7223 bp containing 1037 bp upstream and 1155 bp downstream ORF. The *BcSec3* genomic DNA was obtained by PCR from the B05.10 genome with primer pair BcSec3-com-F(5′-GGACTAGTCCCATAAGTTCGTGCTGTTCG-3′) and BcSec3-com-R(5′-ATAAGAATGCGGCCGCTAAACTATCTCAGCGAATGATAGAGTAAA-3′). The PCR product was cloned into the *Spe* I and *Not* I sites of entry vector pETHG. Then LR recombination reaction between the pETHG-*BcSec3* and binary vector pCAMBIA-Bar-RfA was conducted to create the *BcSec3* gene complementary binary vector p*CAMBIA-Bar-BcSec3* by a Gateway® LRClonaseTM II enzyme cocktail (Invitrogen, USA). The Gateway vectors of pETHG and pCAMBIA-Bar-RfA were kindly provided by Dr. YoKo Nishizawa (Saitoh *et al.* 2008). The resulting binary vector was transformed into *Agrobacterium* AGL-1 for *B. cinerea* spores transformation with the method described before (Cui *et al.* 2015).

2.4 *Reverse transcription PCR*

Total RNA was extracted from *WT, ΔBcSec3-1, 2, 3* and *ΔBcSec3-C* strains after culturing on PDB medium for 3 days by using RNAiso kit (Dalian, Takara). RT-PCR was performed with Abstart One-Step RT-PCR Mix Kit (Shanghai Sangon, China) with primer pair Exon-F(5′-GGAAGAGCAACGCAAGCAA-3′) and Exon-R(5′-TGGCGGAGGCTCATTCTTG-3′). The actin gene was used as a reference to verify the target gene *BcSec3* expression.

2.5 *Mycelial growth, conidiation, and sclerotial formation tests*

For vegetative growth assays, *WT*, *ΔBcSec3*, *ΔBcSec3-C* were first cultured on fresh PDA in dark at 23°C for 5 d. Then a 5 mm diameter mycelial plug was transferred onto the center of a new fresh PDA plate. Radial growth was determined by measuring the colony diameter daily for seven consecutive days at 23°C.

As for conidial yield assay, the method described by Benito et al (1998) was used and modified slightly. The

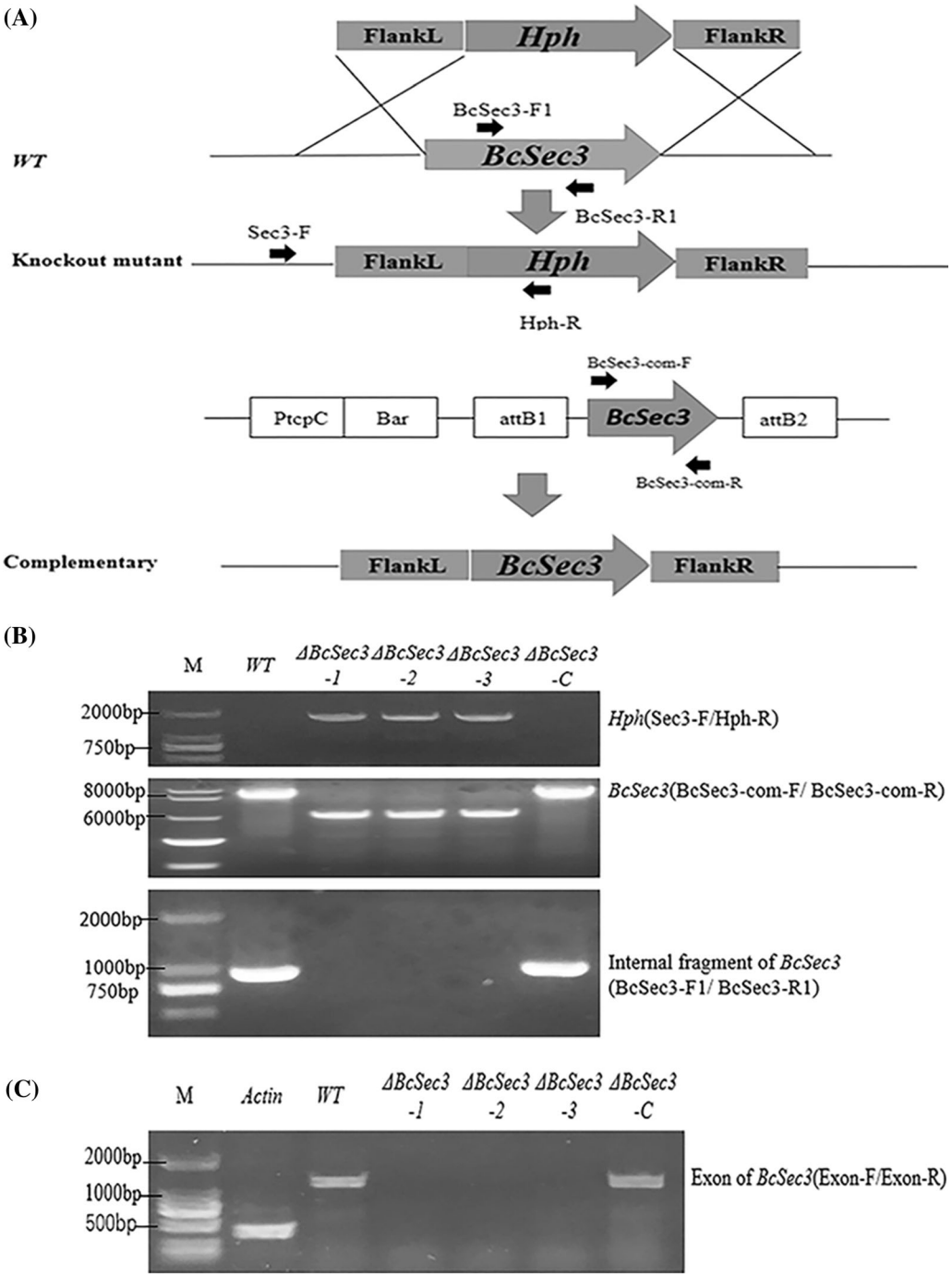

Figure 1. The construction of *BcSec3* deletion mutants. (**A)** Strategy for deletion of *BcSec3*.FlankL, FlankR and hygromycin resistance gene (*Hph*) were used to construct replacement fragment by overlapping extension by PCR. The acquisition of complementary strains was by introducing recombinant *BcSec3* into the knockout by *Agrobacterium* transformation. (**B**) Molecular identification of mutants and complementary strain. *M, Molecular marker. ΔBcSec3-1,2,3, BcSec3 deletion strains. ΔBcSec3-C, complementary* strain. (**C)** *BcSec3* expression test by RT-PCR. Primer pair Exon-F/ Exon-R was used for qualitatively analysis the expression of *BcSec3* in wild-type strain, mutant strains and complementary strain by RT-PCR.

conidia were collected by 3 mL of sterile water on PDA medium cultured for 7 days and filtered through gauze. The conidia concentration was determined using hematocytometer. The sclerotia were counted on PDA medium cultured for 14 days. The vigorously growing mycelium plugs were transferred to PDA plates, cultured at 23°C for sclerotia generation. These test experiment replicated for 3 times

2.6 *Assays for pathogenicity*

To detect pathogenicity, five-day-old mycelial plugs from PDA plates were inoculated onto the upper surface of fresh tomato leaves as described by Liu *et al* (2008). Inoculated leaves were kept in Petri dishes with high humidity under a 12 h light/12 h dark cycle at 23°C for 4 days. Pathogenic conditions were measured daily, with at least three independent experiments for each strain.

2.7 *Observation of cellular vesicles and membrane system*

3-day-old hyphae were cut out from PDA plates and placed at the center of microscope slides carefully. 20 μL of FM4-64 (5 μmol/L) was dropped onto the tip of the hyphae, after which the samples were allowed to stand at room temperature for 10 s. The results were then observed under fluorescence confocal microscope (Zesis, Experimental Center in Zhejiang University) in which the excitation was at 488 nm and emitted light was collected at 514 nm.

2.8 *Ultramicrostructure observation with transmission electron microscope (TEM)*

The strains were cultured in fresh PDA for five days. Hyphae were then collected from the edge of the colonies. The sample was first fixed with 2.5% glutaraldehyde in phosphate buffer and then washed three times in the phosphate buffer. Then the sample was postfixed with 1% OsO_4 in phosphate buffer and washed again. The samples were then washed with a series of gradients of alcohol. Next, the sample was embedded in Spurrresin. The sample was heated at 70°C for more than 9 h. The specimen was sectioned in LEICA EM UC7 ultratome and sections were stained by uranyl acetate and alkaline lead citrate for 5 and 10 min respectively and observed in Hitachi Model H-7650 TEM.

2.9 *Determination of extracellular polygalacturonase activities*

The strains were cultured on fresh PDA for 4 days at 23°C, and equal amount of hyphae were transferred to 100 mL of PDB which were cultured for 3 days at 23°C. 100 μL of the culture solution was used for PG activity measurement by using Polygalacturonase Assay Kit (Solarbio, Beijing). There is a characteristic peak at 540 nm, and this phenomenon can be used to evaluate the activity of polygalacturonase (Jiang *et al.* 2010).

2.10 *Statistics and analysis of data*

The data were displayed as mean ± SD (standard deviation) from three biological replicated experiments or as indicated. The analysis of results was analyzed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). The p values is indicated in figures to show statistical significance.

3. Results

3.1 *The generation of BcSec3 deletion mutants*

The exocyst subunit *BcSec*3 gene was identified at Ensembl (*http://fungi.ensembl.org/Botrytis_cinerea/*. *BcSec3*) gene is only one copy in the genome(Bcin03g04910).The whole gene is 5031 bp long in coding region, with 4 predicted exons and 3 introns. The predicted translation product has 1468amino acids.

In order to analyze the function of *BcSec3* in *B. cinerea,* we used a homologous gene replacement strategy to knockout the target gene (figure 1A). After transformation of protoplasts, the transformants were screened on hygromycin resistant plates and the positive gene deletion mutants were further confirmed using PCR with forward primer Sec3-F(from *BcSec3* upstream) and reverse primer Hph-R(from *Hph* gene), a expected 1755 bp band was obtained in three mutants, but not in *WT*, indicating that the *Hph* gene has been successfully integrated into the *BcSec3* site in genome (figure 1B). And the PCR amplified *Hph* gene was confirmed by sequencing. Three individual mutants (*ΔBcSec3-1, ΔBcSec3-2, ΔBcSec3-3*) were obtained and used for further test experiments. Complementary strains were obtained by *Agrobacterium* mediated transformation and screened on bialophos resistant plates and then confirmed by PCR with primer pair BcSec3-com-F/R (figure 1B). By using primers combination from different location at *BcSec3* in *B. cinerea* genome, characteristic PCR products with different length were used for identification of positive complementary strains. A total of 8

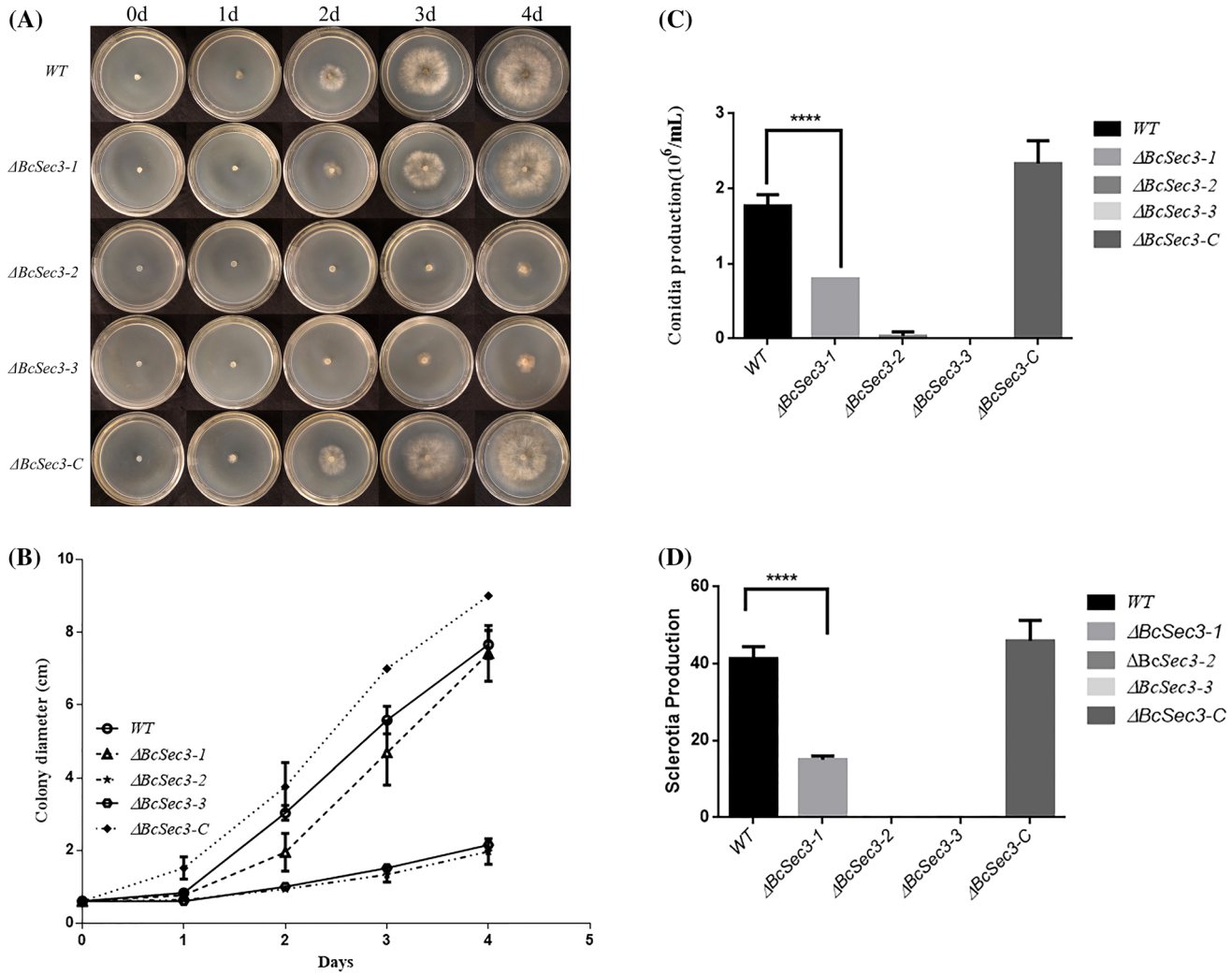

Figure 2. Growth of the *Botrytis cinerea* strains on PDA plates. (**A**) 4-day-old mycelium plugs were transferred to fresh PDA to determine the colony growth. The colony diameters were recorded daily and three experiments were performed in parallel. **(B)** Radial growth rate. Three plates were measured each time. Bars indicate standard deviation of means of three replications. ****$P < 0.0001$. (**C**) The conidium production, mycelia were collected after cultured 7d, followed by washed and filtered. Then conidia were collected. The experiment was repeated three times independently. ****$P < 0.0001$. (**D**) Sclerotia production. Mycelium plugs were transferred to fresh PDA and cultured at 23 °C for sclerotia generation. After 14 days, the sclerotia were counted. Bars indicate standard deviation of means of three replications.

complementary strains were obtained and they were genetically stable. One of them, was named *ΔBcSec3-C,* and chosen for further assay. RT-PCR with primer pair Exon-F/Exon-R was conducted to verify whether the *BcSec3* gene expression was disrupted at the mRNA level in the mutants. The predicted size of exon PCR product was 1036 bp. The results showed that *BcSec3* expressed in *WT* and complementary strain *ΔBcSec3-C*, but not in three mutants (figure 1C). In conclusion, *BcSec3* deletion mutants and its complementary strains were successfully constructed.

3.2 *Deletion of BcSec3 leads to decreased growth, reduced spore production and sclerotia generation of Botrytis cinerea*

The growth of the hyphae of *WT* and mutant strains was assayed in PDA medium. The result indicates that deletion of the *BcSec3* gene resulted in significant decrease in colony growth (figure 2A and B). After 5 day's culture, compared to *WT*, *ΔBcSec3-1*, *ΔBcSec3-2* and *ΔBcSec3-3* decreased in colony diameter by 3.38%, 74.31% and 71.71% respectively. The hyphe of the *ΔBcSec3* strains showed weak and

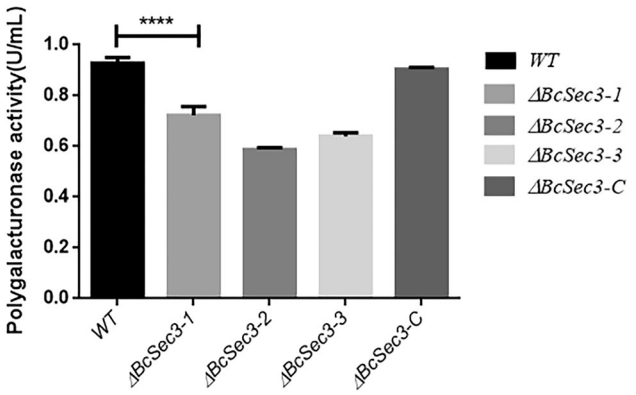

Figure 3. Detection of *Botrytis cinerea* extracellular polygalacturonase activity. The experimental and control groups were repeated three times. ****P < 0.0001.

scattered growth. Microscopical observation found that their hyphae became thinner and no obvious polarized growth was found. Moreover, the sporulation ability in the mutants was drastically impaired. After culturing on PDA for 7 days, there were almost no conidia produced in *ΔBcSec3-2* and *ΔBcSec3-3* (figure 2C). Although the mutant *ΔBcSec3-1* produced conidia, its ability was significantly decreased when compared with *WT*. The sclerotia yields of the mutants were also much less than *WT* (figure 2D). These evidences suggest that *BcSec3* plays an important role in the growth and development of *B. cinerea*. The mutant *ΔBcSec3-2* and *ΔBcSec3-3* showed similar phenotypes. In the case of *ΔBcSec3-1,* its growth rate is higher than *ΔBcSec3-2* and *ΔBcSec3-3*, but significantly lower than *WT* at the 3rd day.

3.3 *Deletion of the BcSec3 results in a decrease in the amount of polygalacturonase(PG) secretion*

PG is one of the enzymes secreted during the infection of *B. cinerea* (Leone *et al.* 1987),which is essential for *B. cinerea* to infect plants. The deletion of the *BcSec3* leads to a decrease in the amount of extracellular PG secretion. When equal amount of hyphae was extracted

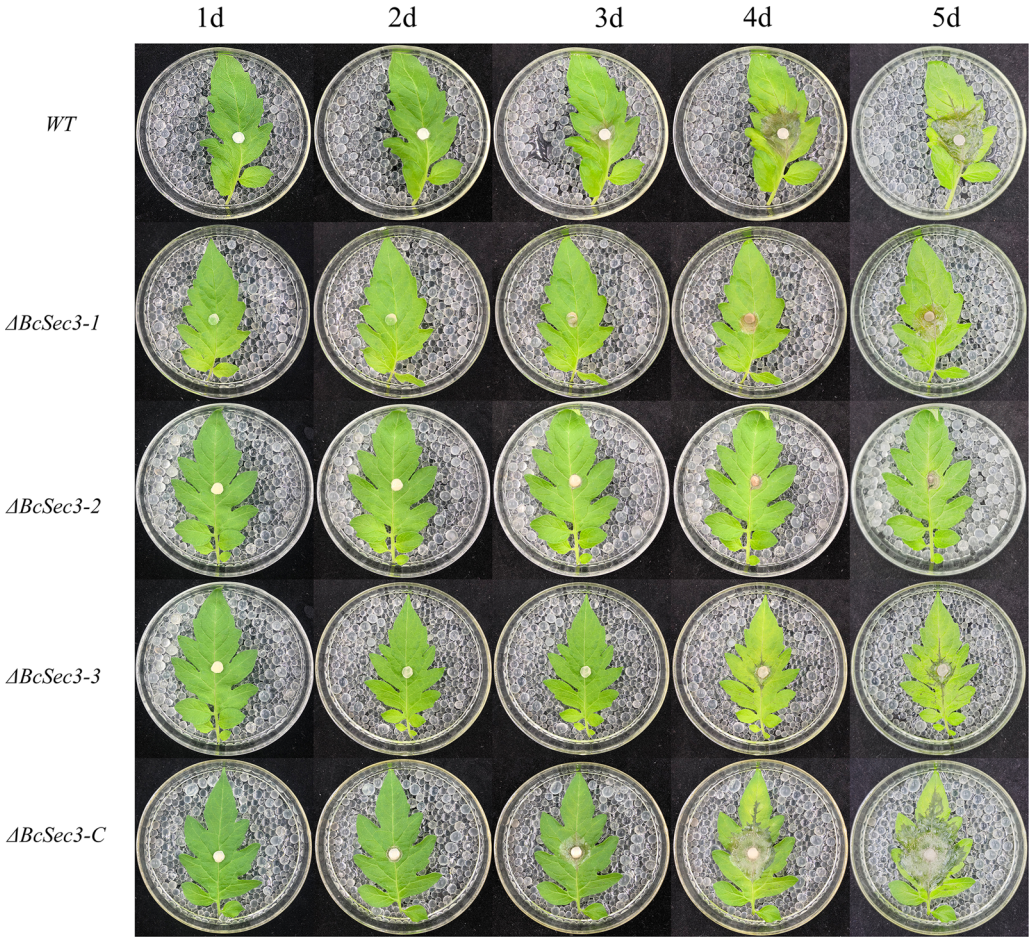

Figure 4. Pathogenicity assays of *Botrytis cinerea* on detached tomato leaves. The 5-day-old mycelial plugs were inoculated onto fresh tomato leaves, with 12 h light and 12 h dark. The environment was kept at 23°C and humid. All strains exist in three parallels, giving similar experimental results.

and tested for PG enzymatic activity, the result found that the PG activity significantly reduced in all three mutants compared with WT, *ΔBcSec3-1* decreased by 22%, *ΔBcSec3-2* decreased by 37%, and *ΔBcSec3-3* decreased by 31% (figure 3).

3.4 *BcSec3 is critical for pathogenicity of Botrytis cinerea*

In order to detect the effect of *BcSec3* on pathogenicity of gray mold caused by *B. cinerea*, the *in vitro* cultured tomato leaves were selected as the infecting host. 4-day-old mycelial discs from PDA were applied to uninjured leaves and the progression of the lesions was monitored daily. The pathogenicity and virulence is directly characterized by the degree of rot and plaque formation in leaf. After 24 h of infection, obvious plaque appeared on the leaves infected by *WT* and *ΔBcSec3-C,* while no plaque appeared on mutans *ΔBcSec3-1, 2, 3*. After 4 days of inoculation, the radial hyphae of the *WT* and *ΔBcSec3-C* strains had almost infected the entire leaf. Incontrast, only slight lesions and normal plant aging appeared on the leaves infected by *ΔBcSec3-1, 2, 3* (figure 4). The evidence provided above indicated that *BcSec3* is critical for virulence of *B05.10.* The deletion of the *BcSec3* cause the loss of pathogenicity of *B. cinerea*.

3.5 *Deletion of BcSec3 prevents fusion of vesicles with plasma membrane*

For detection the function of *BcSec3* in cellular exocytosis, FM4-64 was used to stain lipid structures. In *WT* and *ΔBcSec3-C cell,* the fluorescent signal is mainly concentrated at the tip of the hypha and inner layer of the cell membrane, while in the cytoplasm, a relatively weak fluorescent signal is detected (figure 5). In contrast, in three mutants, the fluorescence signal is mainly concentrated in the cytoplasm, with the fluorescence signal was weaker at the tip of the hyphae. The different location of fluorescence signal indicated the deletion of *BcSec3* results in the failure of the transport and fusion with plasma membrane of vesicles.

3.6 *BcSec3 affects the number of vacuoles and the volume of vesicles*

Filamentous fungi form mycelia and the organelles distributes in cell (Esser 1982). During the growth of

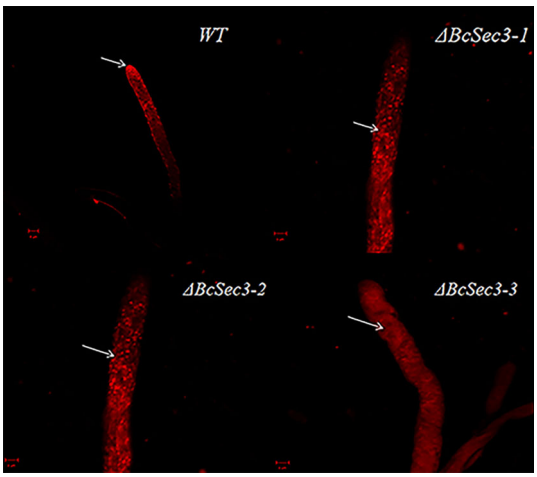

Figure 5. The results of FM4-64 staining phospholipid structure. Transfer the normal-growing mycelium plugs to fresh PDA medium to ensure the vigorous growth of hyphae. Stain the top of the mycelia with 5 mM FM4-64 and image them with confocal microscopy. Cell membranes and vesicles with phospholipid structure are stained red. White arrows in these images indicate aggregation of phospholipid structure.

mycelium, the organelles of mycelium are arranged in order and functional. Here, we have interest to know how the deletion of the *BcSec3* gene gives impact on the vesicles and organelles. Based on TEM observation, we found the mycelial cells of the mutants *ΔBcSec3-1, 2, 3* are enlarged, and the number of vacuoles in mutants increased when compared to *WT* (figure 6). The volume of vesicles in the *ΔBcSec3-1, 2, 3* are larger than that in the *WT. M*oreover, corresponding to the FM4-64 staining results, the vesicles in the *ΔBcSec3-1, 2, 3* mostly accumulated in the cytoplasm, while the vesicles of in WT and complementary strain *ΔBcSec3-C* were mostly located inside the cell membrane. These evidences suggest that the deletion of *BcSec3* can lead to abnormal cell growth and disability to direct vesicle secretion, which maybe play a function of delivering substances involved in polarized hyphal growth and virulent factors for pathogenesis process.

4. Discussion

In plant pathogenic fungi, there is increasing evidence that exocyst plays an important role in biology and pathogenicity of plant pathogenic fungi. The functions of exocyst subunits are gradually being revealed. Our previous research demonstrated that exocyst subunit *BcExo70* in *B. cinerea* is involved in growth, development and pathogenicity to tomato (Guan *at al.*

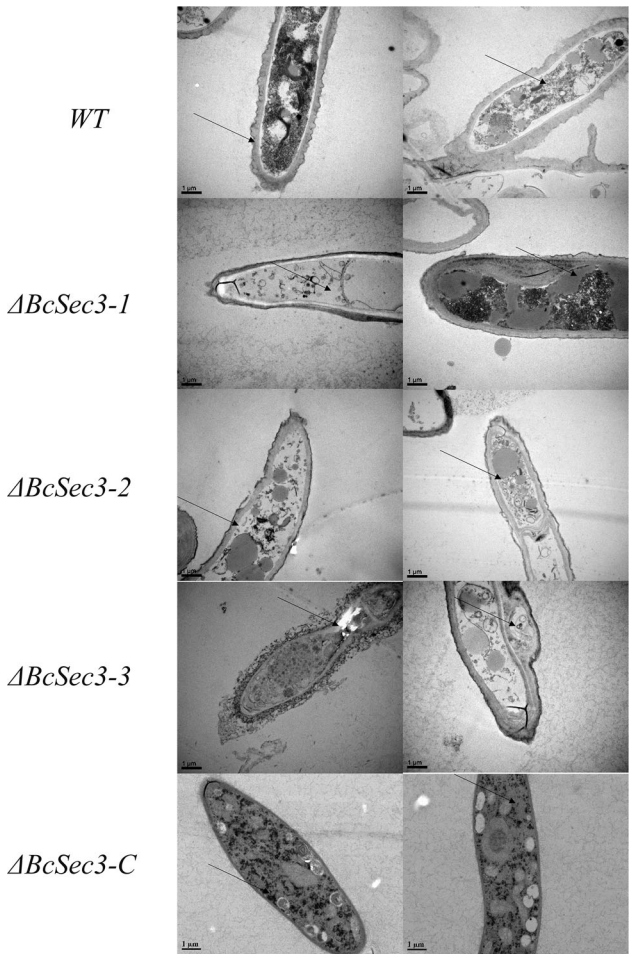

Figure 6. The results of electron microscopic observation for mycelium tip cell. Two representative pictures are provided for each strain. The scale bars represent 1 μm. The arrow points to the vesicle structures. In *WT* and *ΔBcSec3-C*, vesicles mainly distributed near the cell membrane, while in *BcSec3* mutants, *ΔBcSec3-1,2,3,* vesicles of mainly concentrated in the cell matrix.

2020). In yeast and other fungi, as one of the eight subunits of exocyst, Sec3 has a unique function and is different from other subunits. However, the function of *BcSec3* in *B. cinerea* is largely unknown.

Exocyst is a conserved protein complex. Although most of the putative plant exocyst genes exist as multiple copies, animals and fungi often have only one copy of each subunit (Elias *et al*. 2003; Synek *et al*. 2006; Cvrckova *et al.* 2012). Therefore, exocyst subunits are decisive for exocytosis in fungi. At the same time, because the subunit genes exist as a single copy, it is easier to knock out exocyst genes and analyze their function. The genomic data of *B. cinerea* showed that it contained a copy of the putative Sec3 gene, and we used the method of homologous recombination to knock it out, which proved that the BcSec3 subunit plays the similar role as in yeast.

Exocytosis is essential for cell growth and polarization in all eukaryotes. In general, the polarized growth of fungi depends on the exocytosis of cells. In polarized exocytosis, the vesicles carrying proteins dock on the plasma membrane and then fuse with each other (Zhang *et al*. 2019). In this case, the mycelium has completed the extension to the outside. In yeast, vesicles are located at the growth end of the daughter cells ('bud tips') (He and Guo 2009). As mentioned above, the function of exocytosis is closely related to exocyst. It is clear that exocyst are essential for the growth and development of fungi, and mutations or deletions of exocyst subunits can cause cells to fail to grow normally (Kwon *et al*. 2014; Riquelme *et al*. 2014; Chavez-Dozal *et al*. 2015). Our research shows that the deletion of *BcSec3* seriously weaken the basic biological functions of *B. cinerea*, which leads to the sparse and scattered mycelial growth and loss of its ability to grow polarized.

Multiple evidences suggest that the absence of exocyst subunits can lead to the accumulation of Golgi-secreting vesicles in cells (Chavez-Dozal *et al.* 2015; Tang *et al*. 2019). Research on the fungal Sec3 subunit in yeast cells have demonstrated that its main function is targeted transport and tethering of vesicles (Luo *et al*. 2014). The post-Golgi vesicles unload the 'cargo' at the correct location positioned by Sec3. With the exception of Exo70 and Sec3, the remaining subunits of exocyst are directly related to vesicles and rely on the actin cable to transport them to the site of exocytosis (Boyd *et al*. 2004; Bendezú *et al*. 2012). As to the assembly of exocyst, Sec5 plays an important role. Sec5 binds to mature secretory vesicles and then recruits additional subunits for assembly (Mizuno-Yamasaki *et al.* 2010; Guo *et al*. 1999). During the docking of vesicles and plasmalemma, vesicles are controlled by various small G proteins and need SNARE(sensitive factor-attachment protein receptor) complex to mediate this process, in which exocyst are responsible for ensuring the precise location of vesicles, including time and location (Terbush *et al*. 1996; Wu *et al*. 2008).It was demonstrated that Sec3 mediates vesicle tethering during exocytosis and plays a key function in activation of SNARE complex assembly by directly interacting with the t-SNARE protein Sso2 (Yue *et al*. 2017).

The evidences for understanding of fine molecular mechanism of how exocyst interact with cell membrane were found in the study of subunit Sec3 in yeast. Sec3 was shown directly interacts with phosphatidylinositol 4,5-bisphosphate with its N terminus, which

explains well the targeted physical association of exocyst with the plasma membrane. The interactions of Sec3 with phospholipids and Cdc42 was also proposed that play important roles in exocytosis and polarized cell growth (Zhang *et al*. 2008; Bloch *et al*. 2016).Our study do not provide enough evidences that BcSec3 directly regulates vesicle secretion, but in the mutant cells without *BcSec3*, laser confocal and ultra-thin section electron microscopic observation showed that vesicles were mainly scattered in the cytoplasm and failed to successfully target the cell membrane, suggesting that *BcSec3* may play a critical role in vesicle targeting and tethering, similar to yeast Sec3.

For filamentous fungi, vesicles secreted by the Golgi apparatus and then targeted by exocyst not only carry substances required for cell growth, but also carry hundreds of pathogenic-related factors such as effector proteins used to infect plants (Mosquera *et al*. 2009; Bleackley *et al*. 2020). In this case, exocyst is particularly important for disease occurrence because the vesicles may carry plant cell wall degrading enzymes that can promote infection. Polygalacturonase and xylanase have been shown to be pathogenic factors related to the virulence of *B. cinerea* (ten Have *et al*. 1998; Yang *et al*. 2018). Pectin would form galacturonic acid under the hydrolysis of polygalacturonase, resulting in producing a reducing aldehyde group which is able to react with DNS reagent to form a reddish brown substance. Our research found that the polygalacturonase activity of *ΔBcSec3* was significantly reduced, and the corresponding pathogenicity was much lower than that of wild-type strain. It is most likely due to the failure of exosome assembly, which leads to the inhibition of vesicle transport and the inability to secrete pathogenic factors. The production of spores and sclerotia requires the participation of melanin, which is also a pathogenic factor (Chen *et al*. 2004; Doss *et al*. 2003). The phenotype of significant decrease of sclerotia production in *ΔBcSec3* mutant could be explained partially by the loss of melanin supply via vesicle transport.

In eukaryotic cells, exocyst are multi-protein complexes that are functionally and structurally conserved and are essential for many basic biological processes (Doss *et al*. 2003; Chou *et al*. 2016). But so far, little research has been done on exocyst in *B. cinerea*. Our results indicate that *BcSec3* is essential for *B. cinerea* growth, development and pathogenicity. However, exocyst and its related processes are very complicated, and there are more proteins and other related factors involved. The interaction between them and the molecular mechanisms underlying exocyst-medaited vesicle secretion and exocytosis remains to be further studied in *B. cinerea*.

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