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The small GTPase BcSec4 is involved in conidiophore development, membrane integrity, and autophagy in *Botrytis cinerea*

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Abstract

Small GTPases of the Rab family play important roles in membrane trafficking and autophagy. Previous studies have shown that the small GTPase Sec4 is involved in vegetative growth, protein secretion, and pathogenicity of fungal phytopathogens. In this study, the Rab GTPase BcSec4, an ortholog of the yeast Sec4p protein, was characterized in *Botrytis cinerea*. The *ΔBcSEC4* mutant produces many abnormal conidiophores; it is thus defective in conidiation and displays attenuated pathogenicity. Notably, the germination and penetration of conidia are unaffected in the *ΔBcSEC4* mutant. Further studies showed that the secretion of extracellular proteins was altered in the *ΔBcSEC4* mutant, and the mutant showed impaired autophagy and defects in membrane integrity. Taken together, these results suggest that BcSec4 is critical for conidiophore development, membrane integrity, pathogenicity, and autophagy.

Keywords: BcSec4, *Botrytis cinerea*, Conidiophore morphology, Membrane integrity, Autophagy, Pathogenicity

Background

Botrytis cinerea causes serious crop disease that is responsible for significant economic losses (Williamson et al. 2007; Fillinger and Elad 2016). As a necrotrophic fungal phytopathogen, it uses numerous virulence factors as weapons to attack host plants during the infection process, including extracellular enzymes, effectors, and phytotoxins secreted into plant cells (Staples and Mayer 1995). These virulence factors are transported to the extracellular space by secretory vesicles; they have necrotizing activity and are involved in the degradation of different plant structures (Kan et al. 1997; Espino et al. 2005; Brito et al. 2006; Kars et al. 2010; Schouten et al. 2010).

Vesicle trafficking is essential for multiple biological processes in eukaryotes including plant pathogens. In *Saccharomyces cerevisiae*, the Rab family of GTPases have important functions as regulators of vesicle trafficking, including vesicle docking and fusion, and play an important role in extracellular protein secretion pathways (Schmitt et al. 1986; Salminen and Novick 1987). Rab proteins also play important roles in autophagy, and abundant evidence indicates that numerous Rab proteins are involved in various stages of autophagy in yeast and mammals. Yeast Ypt1, which is homologous to human Rab1, was first found to be involved in autophagosome formation (Tran et al. 2016). Subsequently, several additional Rab proteins (Rab5, Rab7, Rab8B, Rab11, Rab24, Rab32, and Rab33B) were shown to participate in autophagosome formation and maturation (Tsukada and Gallwitz 1996; Knoedler et al. 2010; Ao et al. 2014; Zheng et al. 2015; Liu et al. 2016; Yang et al. 2020). Sec4, whose human homolog is Rab8, participates in autophagy as a GTPase and is diverted to direct membrane flow to autophagosome formation (Geng et al. 2010).

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Sec4 regulates the late stage of protein secretion, contributing to the transport of vesicles from the post-Golgi to the plasma membrane (Bastos 2008). Other Sec4 homologues in plant pathogens have been reported to contribute to various cellular functions and pathogenicity. In *Colletotrichum lindemuthianum*, the Rab GTPase Clpt1, homologous to *S. cerevisiae* Sec4, was identified to participate in vesicular trafficking. The *Clpt1*-deleted mutant showed impaired protein secretion and pathogenicity (Dumas et al. 2001). *Fusarium graminearum* FgRab8 regulates vegetative growth and hyphal morphology and its deletion mutant showed reduced conidiation, premature death, and reduced germination (Zheng et al. 2015). In *Fusarium verticillioides*, the *FvSEC4* mutant exhibited defects in virulence and a significant alteration in mycotoxin production (fumonisin B1) (Yan et al. 2019). In *Aspergillus fumigatus*, a mutant of the Sec4 homolog SrgA exhibited defects in stress response, attenuated virulence to the insect *Galleria mellonella*, and phenotypic heterogeneity (Powers-Fletcher et al. 2013). MoSec4 was shown to participate in hyphal development, secretion of extracellular effectors and pathogenicity in *Magnaporthe oryzae* (Zheng et al. 2016).

Although Sec4 is known to be essential for protein secretion in some plant pathogens, the function of BcSec4 in the pathogenic fungus *B. cinerea* has not been fully characterized (Zhang et al. 2014b). We here present the functional characterization of BcSec4 in *B. cinerea* and our results indicate that BcSec4 is required for fungal morphogenesis, membrane integrity, autophagy, stress resistance, and pathogenicity in *B. cinerea*.

Results

BcSec4 is required for vegetative growth and conidiogenesis in *B. cinerea*

To identify the function of *BcSEC4* in *B. cinerea*, the *ΔBcSEC4* mutants were generated using a hygromycin resistance gene as a selectable marker. We cultured B05.10, the *ΔBcSEC4* mutants, and complemented strains on potato dextrose agar (PDA) medium at 25 °C for 4 days. The *ΔBcSEC4* mutants showed a significant reduction in colony size compared with B05.10 and complemented strains (Additional file 1: Figure S1a). The *ΔBcSEC4* mutants also displayed more hyphal branching and greater colony density compared with B05.10 under microscopic examination (Additional file 1: Figure S1b). Hyphal tips of the *ΔBcSEC4* mutants displayed abnormal morphology and were prone to swelling (Additional file 1: Figure S1c).

To further evaluate the role of BcSec4 in conidial production, we inoculated mycelial plugs of B05.10, the *ΔBcSEC4* mutants, and complemented strains onto PDA plates and assessed conidiation ability of these strains at 10 days post-incubation. The *ΔBcSEC4* mutants produced significantly fewer conidia compared with B05.10 and complemented strains (Fig. 1a, b). Consistent with this result, we observed that conidiophore morphogenesis was altered in the *ΔBcSEC4* mutants (Fig. 1c). Forty percent of the conidiophores were crinkled and did not form conidia, and 14% of the conidiophores formed new mycelium (Fig. 1d). These results indicated that deletion of *BcSEC4* caused a defect in sympodial conidiogenesis and a subsequent reduction in conidial production. Taken together, these results suggest that BcSec4 plays important roles in conidiogenesis of *B. cinerea*.

To test whether BcSec4 mediates conidial germination, we evaluated conidial germination of B05.10, the *ΔBcSEC4* mutants, and complemented strains in yeast extract peptone dextrose (YEPD) liquid medium or on PDA plates. Over a 12 h of incubation, conidial germination and germling development did not differ significantly among the tested strains (Fig. 1e, f). This result indicated that BcSec4 is dispensable for conidial germination. However, after 48 h of incubation on PDA plates, mycelial growth of the *ΔBcSEC4* mutant was affected, and its colony diameter was smaller than that of B05.10. These data demonstrate that deletion of *BcSEC4* did not affect conidial germination and germ tube development of the mutant strains, but attenuated the mycelial development of *B. cinerea*.

BcSec4 plays an important role in response to various stressors in *B. cinerea*

In *S. cerevisiae*, Sec4p is involved in responses to environmental stress. To examine the role of BcSec4 to different stressors, we investigated the mycelial growth of B05.10, the *ΔBcSEC4* mutants, and complemented strains on media containing several stressors, including Congo red (CR, cell wall stress), sodium dodecyl sulfate (SDS, cell membrane stress) and H_2O_2 (oxidative stress). Mycelial growth of the *ΔBcSEC4* mutants was more tolerant to media containing CR, SDS, and H_2O_2 compared with that of B05.10 and complemented strains (Fig. 2a). The growth inhibition rates of the *ΔBcSEC4* mutants in response to CR, SDS, and H_2O_2 significantly decreased compared with those of B05.10 and complemented strains (Fig. 2b). This result suggests that BcSec4 is involved in cell wall integrity and in tolerance to oxidative stress.

BcSec4 mediates the production of acidic compounds

B. cinerea can produce organic acids to affect its ambient environment. To investigate their acidification ability, B05.10, the *ΔBcSEC4* mutants, and complemented strains were examined on PDA medium using the pH indicator bromothymol blue. B05.10 and complemented strains normally acidified its environment,

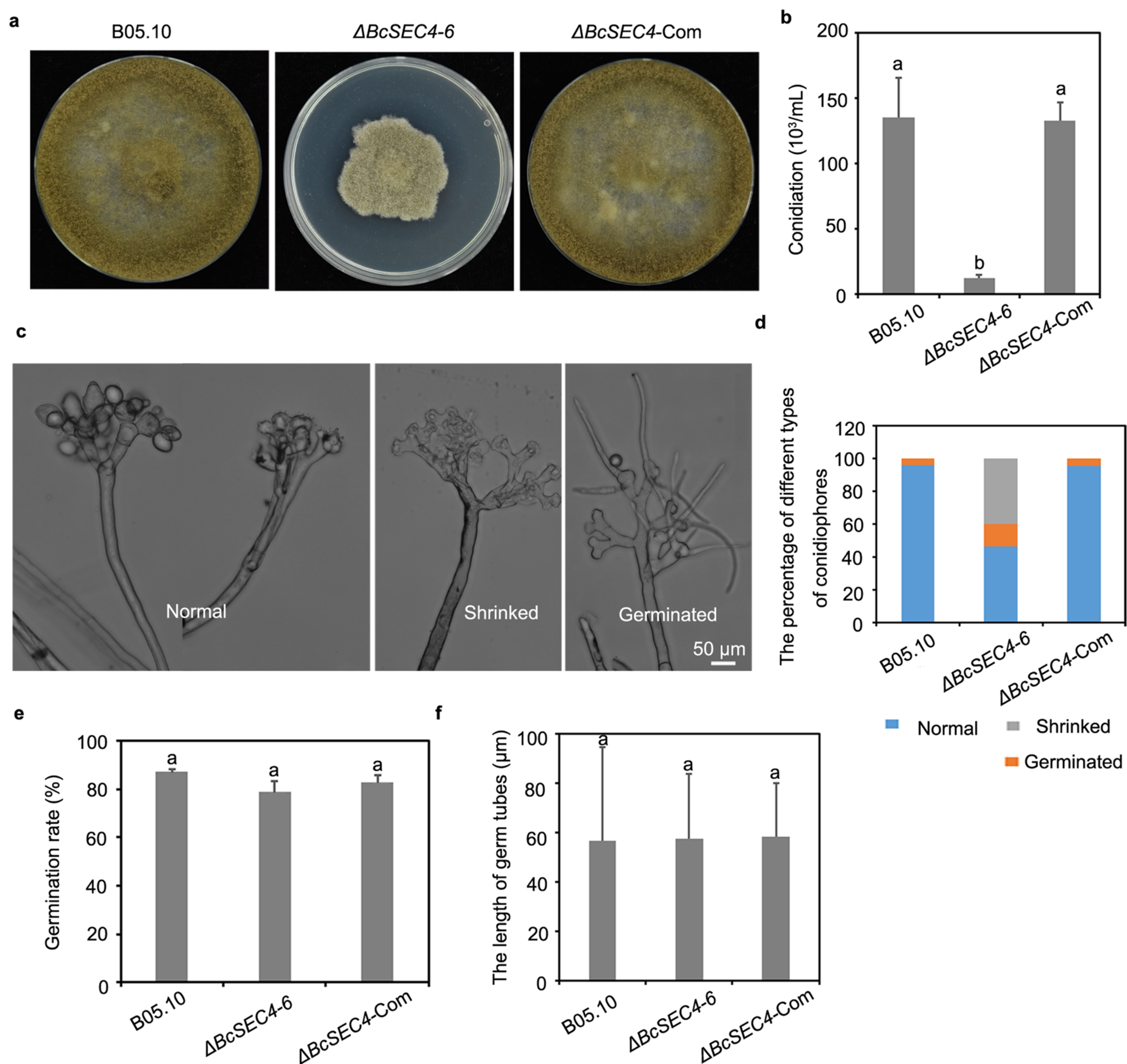

Fig. 1 BcSec4 is required for conidiation of *B. cinerea*. **a** Conidial production of B05.10, the *ΔBcSEC4* mutant, and complemented strains observed on PDA plates at 25 °C for 7 days. **b** Conidiation capacity of the indicated strains. Conidia were harvested from PDA plates after incubation for 7 days at 25 °C. **c** Conidiophore morphology of the *ΔBcSEC4* mutant on PDA plates for 7 days. **d** Percentage of abnormal conidiophores produced by the indicated strains. More than 100 conidiophores of each strain were measured in each experiment. **e** Germination rates examined under a microscope after 12 h of incubation in YEPD liquid medium. **f** The lengths of germ tubes examined after 12 h of incubation in YEPD liquid medium. All data are represented as means ± standard deviations (SDs) from three independent experiments with triplicate slides examined for each treatment. Different letters indicate statistically significant differences ($P < 0.01$)

causing the medium to turn yellow, but the *ΔBcSEC4* mutants showed reduced acidification (Fig. 3a). The pH change in liquid medium (YEPD) was also determined at 48 h post-inoculation (hpi), and the medium pH of the *ΔBcSEC4* mutant was higher than that of B05.10 (Fig. 3b).

BcSec4 participates in the secretion of extracellular enzymes

To investigate whether BcSec4 participates in the secretion of extracellular proteins, we examined B05.10, the *ΔBcSEC4* mutants and complemented strains on PDA culture medium containing 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), a well-known laccase mediator (Chen et al. 2015). We found that the culture medium of the *ΔBcSEC4* mutants showed a significantly stronger purple color compared with that of B05.10 (Fig. 4a). When the *ΔBcSEC4* mutants and B05.10 were cultured on V8 medium, the mutants showed a clear transparent circle which did not appear for B05.10 (Fig. 4b). These results suggested that *BcSEC4* deletion partially promoted the secretion of extracellular proteins.

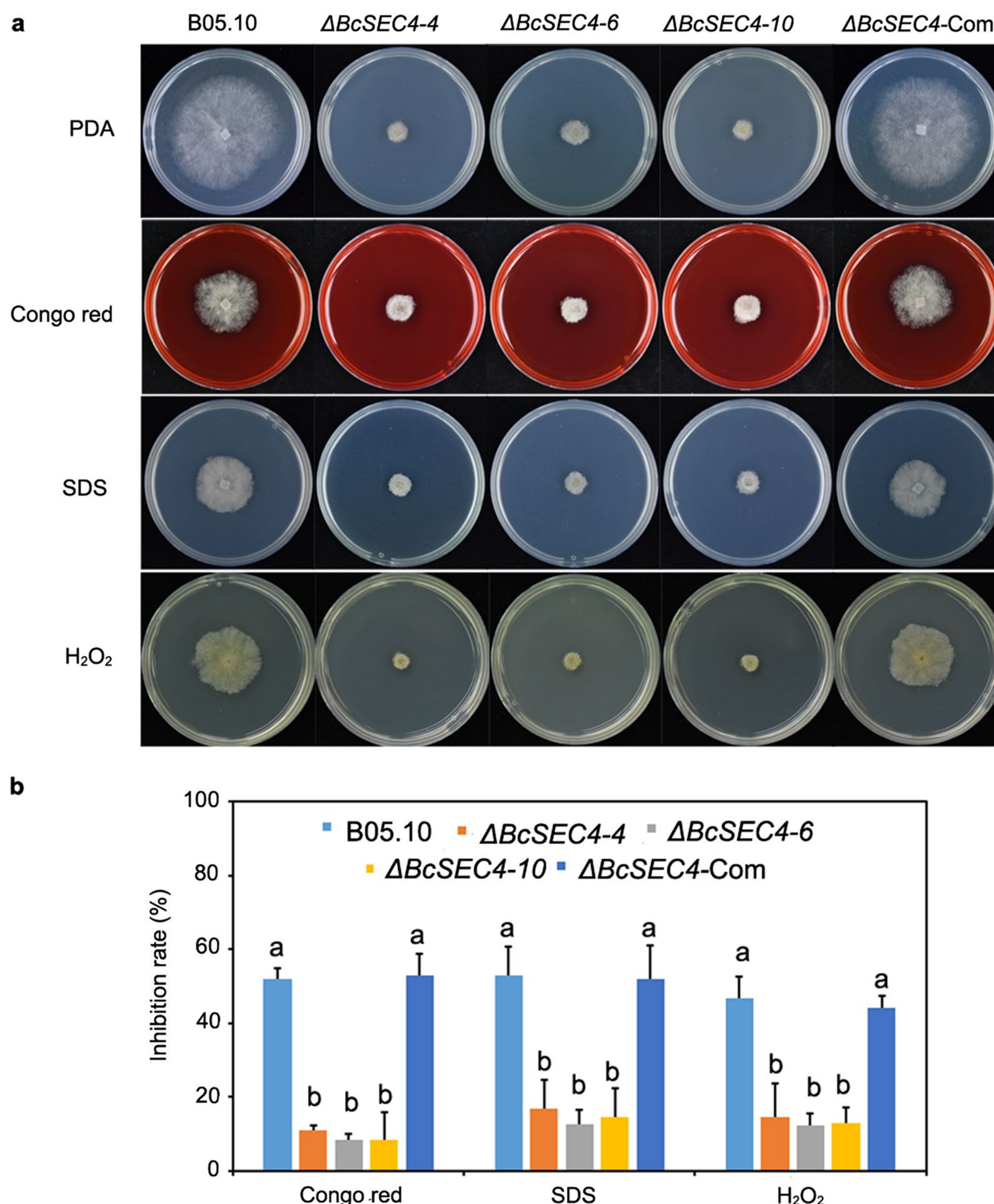

Fig. 2 BcSec4 plays important roles in response to various stressors in *B. cinerea*. **a** The wild-type strain B05.10, the *ΔBcSEC4* mutants, and complemented strains grown on PDA medium with or without stress inducers as indicated for 3 days at 25 °C. **b** Inhibition rates of various stressors on vegetative growth of the indicated strains. The growth inhibition rate (%) = [(colony diameter without stressor − colony diameter with stressor)/(colony diameter without stressor)] × 100. Three replications were performed with similar results. Different letters indicate statistically significant differences ($P < 0.01$)

We also determined the necrotic capacity of the culture supernatant, and culture supernatant from that of B05.10 caused larger necrotic areas on *Nicotiana benthamiana* than that of the mutant (Additional file 1: Figure S2a).

The secretomes of B05.10 and the *ΔBcSEC4* mutant were characterized and compared. B05.10 and the *ΔBcSEC4* mutant were cultured in YEPD liquid medium for 24 h, and extracellular proteins were then obtained from the supernatant. The extracellular proteins were analyzed by SDS-PAGE and silver staining. Obvious differences were observed between the proteins from the *ΔBcSEC4* mutant and B05.10 (Additional file 1: Figure S2b). The extracellular proteins were then analyzed by tandem mass spectrometry

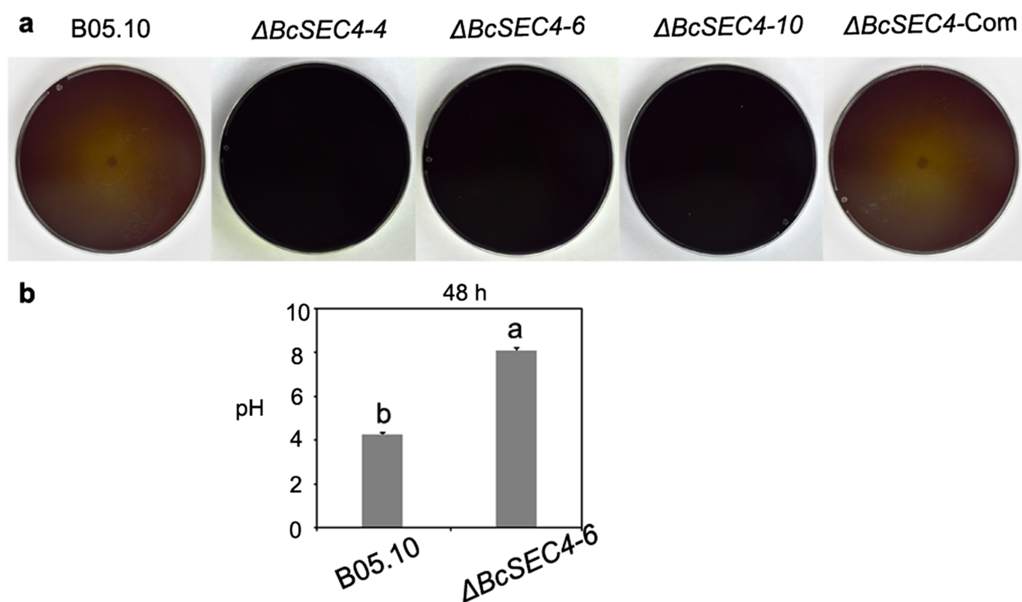

Fig. 3 BcSec4 is required for acidogenicity of *B. cinerea*. **a** Medium acidification by B05.10, the *ΔBcSEC4* mutants, and the complemented strain. The tested strains were grown on solid complete medium supplemented with a pH indicator. The color change from purple to yellow indicates acidification (pH < 6.0). **b** PH value determined after incubated in YEPD liquid medium for 48 h. Data are represented as means ± standard deviations (SDs) from three independent experiments. Different letters indicate statistically significant differences ($P < 0.01$)

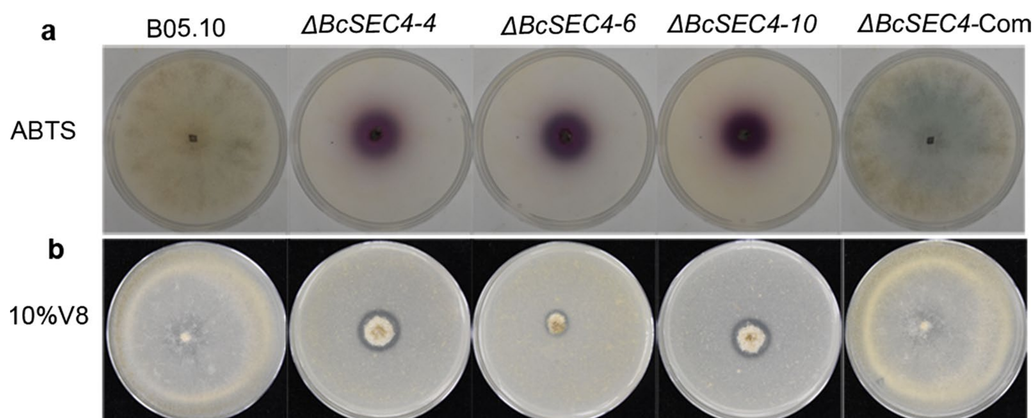

Fig. 4 Deletion of *BcSEC4* promotes the secretion of extracellular proteins of *B. cinerea*. **a** Laccase activity tested on PDA medium containing ABTS and photographed after 4 days of incubation at 25 °C. **b** A clear zone formed on 10% V8 medium and photographed after 6 days of incubation at 25 °C

(MS/MS): 15 expressed proteins were detected in the secretome of B05.10, and 20 expressed proteins were detected in that of the *ΔBcSEC4* mutant. The amount of cerato-platanin secretion was significantly reduced in the *ΔBcSEC4* mutant compared with B05.10, whereas glycosyl hydrolase family protein, esterase, galactose oxidase protein, and laccase were significantly increased in the *ΔBcSEC4* mutant (Additional file 1: Figure S2c).

Deletion of *BcSEC4* attenuates the pathogenicity of *B. cinerea*

The pathogenicity of B05.10, *ΔBcSEC4* mutants, and complemented strains was determined via the assessment of average diameter of lesions on detached leaves of tomato (*Solanum lycopersicum* cv. Ailsa Craig) and mung bean (*Vigna radiata*) inoculated with conidial suspension. At 3 days post-inoculation (dpi), B05.10 and complemented strains caused lesions. By contrast, the

ΔBcSEC4 mutants did not produce obvious lesions on detached tomato leaves (Fig. 5a, b). Similar results were obtained in mung bean leaves (Fig. 5c, d). In mycelial plug inoculation, lesions caused by all the tested strains were observed at 3 dpi, with the *ΔBcSEC4* mutant causing smaller lesions on detached tomato and mung bean leaves at 3 dpi (Additional file 1: Figure S3). We speculated that the loss of pathogenicity in the *ΔBcSEC4* mutant may have been due to the absence of infection cushion formation. However, the results showed no significant differences in infection cushion formation between B05.10 and the *ΔBcSEC4* mutant (Additional file 1: Figure S4a, b).

To further understand the attenuated pathogenicity of the *ΔBcSEC4* mutants, an onion epidermal cell infection assay was used to analyze the host penetration of the strains. Both B05.10 and the *ΔBcSEC4* mutant conidia could penetrate the onion epidermis at 12 hpi (Fig. 5e, f). Further examination revealed that invasive hyphae of the *ΔBcSEC4* mutant were shorter than that of B05.10 (Fig. 5g). We also investigated hyphal expansion in onion epidermal cells at 36 hpi: invasive hyphae of both B05.10

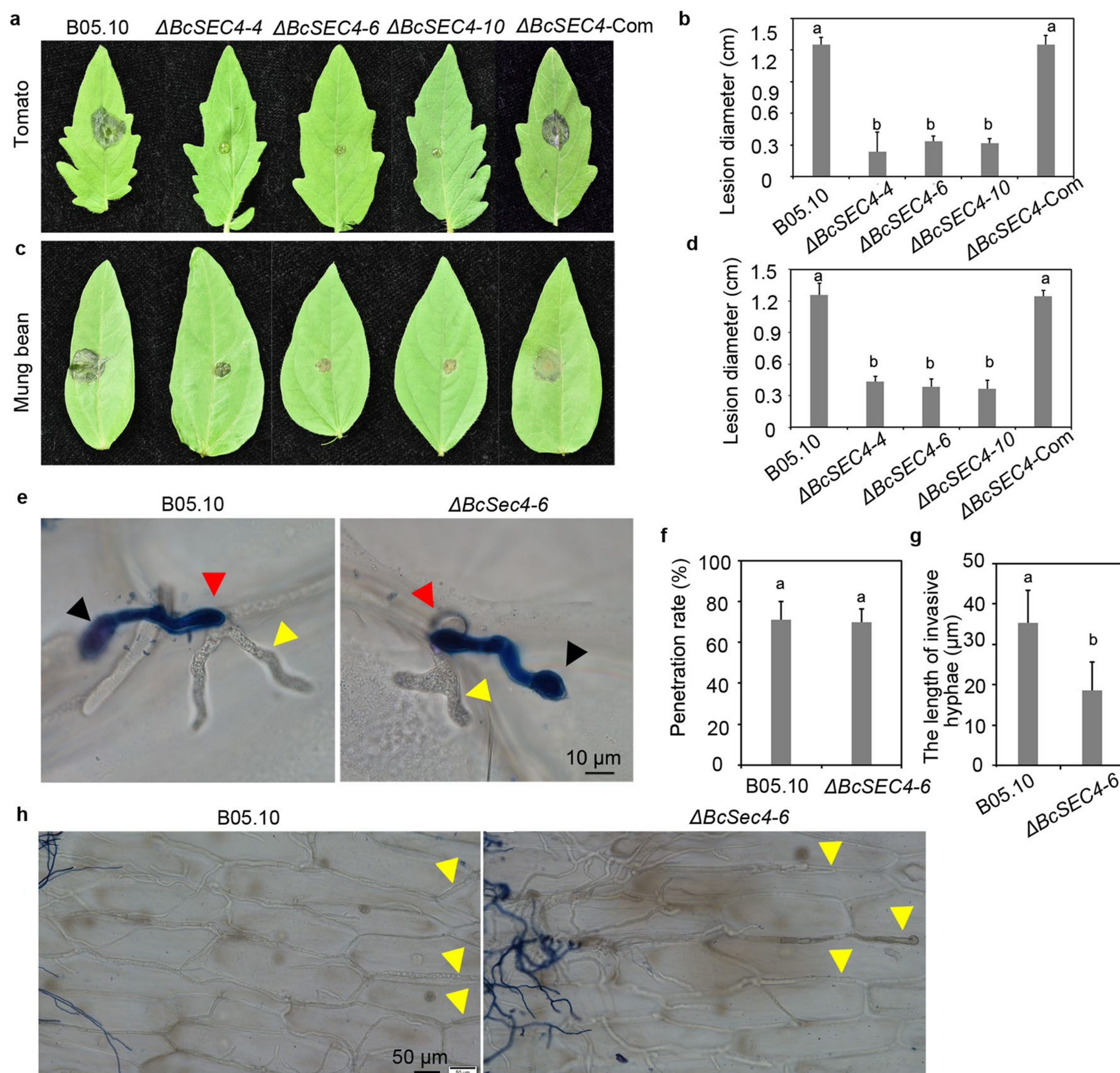

Fig. 5 *BcSEC4* is required for full virulence of *B. cinerea*. Phenotypes of tomato (**a**) and mung bean leaves (**b**) after drop-inoculated with conidial suspensions (1 × 10^5 conidia/mL) of B05.10 and the *ΔBcSEC4* mutant for 3 days. Column diagram showing lesion size on leaves of tomato (**c**) and mung bean (**d**) corresponding to **a** and **b**. **e** Penetration assay performed on onion epidermis and observed under a microscope at 12 hpi. Red arrows: appressoria; Black arrows: conidia; Yellow arrows: invasive hyphae. **f** Relative penetration rate on onion epidermis at 12 hpi. At least 50 infection hyphae were counted for each strain. **g** The lengths of invasive hyphae measured at 12 hpi. **h** The expansion of B05.10 and the *ΔBcSEC4* mutant in onion epidermal cells (yellow arrows) observed under a microscope at 36 hpi. All data are represented as means ± standard deviations (SDs) from three independent experiments. Different letters indicate statistically significant differences ($P < 0.01$)

and the *ΔBcSEC4* mutant exhibited normal expansion into the onion epidermis. However, B05.10 exhibited much faster invasive hyphal growth and expansion in onion epidermal cells compared with the *ΔBcSEC4* mutant (Fig. 5h). As mentioned earlier, deletion of *BcSEC4* affected protein secretion. To explore the defense response in tomato after inoculation with B05.10 and the *ΔBcSEC4* mutant, the expression of defense-related genes induced by B05.10 and the *ΔBcSEC4* mutant at 12 hpi was analyzed. The expression of JA-related genes (*SlPI-I*, *SlPI-II*, *SlMYC-2*, *SlJA1*, *SlTD2*, and *SlWRKY33*) was significantly up-regulated in tomato leaves inoculated with *ΔBcSEC4* mutant compared with those inoculated with B05.10 (Additional file 1: Figure S5). Taken together, the impaired development of invasive hyphae and the up-regulation of defense genes resulted in reduced pathogenicity of the *ΔBcSEC4* mutant.

Deletion of *BcSEC4* disrupts the plasma membrane integrity in *B. cinerea*

Sec4 is involved in the transport of vesicles from the trans-Golgi to the plasma membrane. We therefore investigated the integrity of the plasma membrane in the *ΔBcSEC4* mutants by staining with PI, a cell-membrane-impermeable dye (Ma et al. 2020). After 30 min of treatment, PI-stained nuclei were observed in the *ΔBcSEC4* mutants. However, very few nuclei were stained by PI in B05.10 (Fig. 6a). These results suggest that the plasma membrane of the *ΔBcSEC4* mutant was compromised. Dichlorofluorescein diacetate (DCFH-DA) is commonly used to detect ROS generation and as a membrane-permeable probe. It crosses the cell membrane and is deacetylated by intracellular esterases to form DCFH, which is retained intracellularly and cannot cross the cell membrane. Nitric oxide, superoxide and hydrogen peroxide convert DCFH to the highly fluorescent DCF (Hua et al. 2019). The mycelium of B05.10 produced stronger fluorescence after 30 min of DCFH-DA treatment compared with that of the *ΔBcSEC4* mutant (Fig. 6b). However, the fluorescence of the supernatant was stronger for the *ΔBcSEC4* mutant than for B05.10 (Fig. 6c), suggesting that the cell membrane of the *ΔBcSEC4* mutant may have been compromised.

To further investigate changes in the plasma membrane of the *ΔBcSEC4* mutant, we performed a morphological analysis using transmission electron microscopy. The partial plasma membrane of the *ΔBcSEC4* mutant folded into a double membrane, whereas no double membrane was observed for B05.10 (Fig. 6d). We also quantified membrane integrity using electrical conductivity. Compared with B05.10, the *ΔBcSEC4* mutant showed increased electrical conductivity in YEPD culture

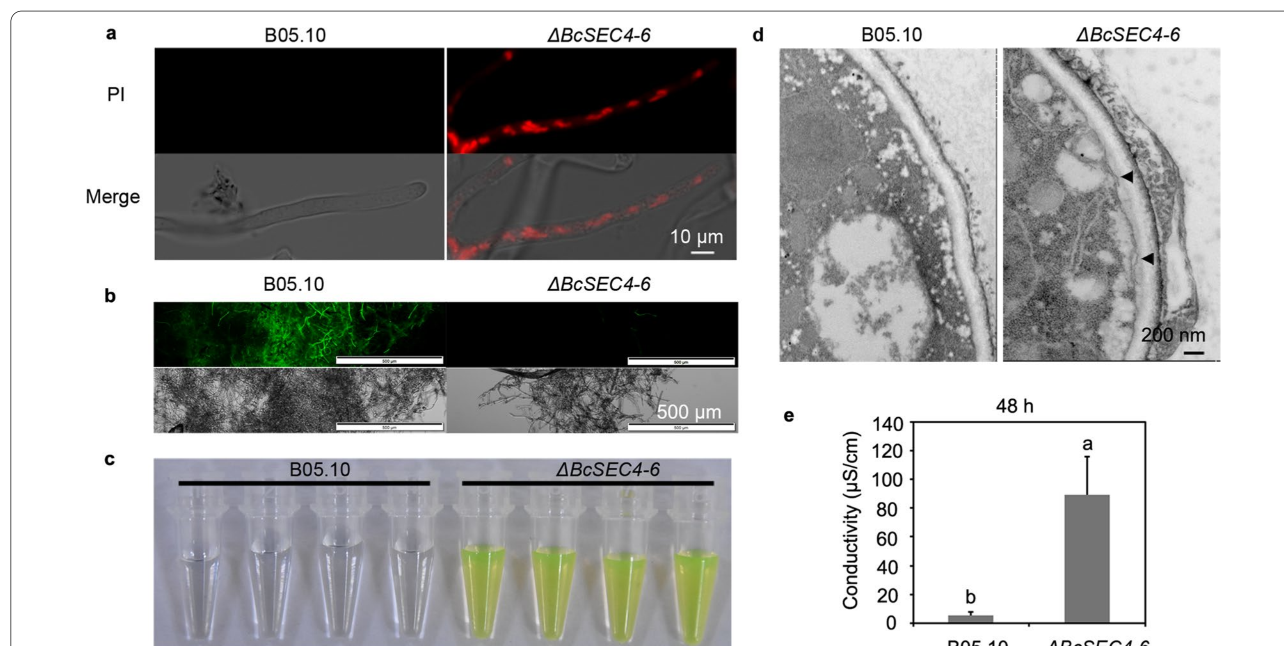

Fig. 6 BcSec4 is required for membrane integrity of *B. cinerea*. **a** Plasma membrane integrity of B05.10 and the *ΔBcSEC4* mutant assessed by staining with the small molecule propidium iodide (PI). **b** ROS accumulation assessed by DCFH-DA staining. **c** Fluorescence accumulation in the supernatant of conidial suspension. **d** Transmission electron microscopy observation of the plasma membranes of B05.10 and the *ΔBcSEC4* mutant cultured in YEPD liquid medium for 12 h. Arrowhead indicates a site of cell membrane damage. **e** The conductivity of B05.10 and the *ΔBcSEC4* mutant measured after inoculation for 24 h. All data are represented as means ± standard deviations (SDs) from three independent experiments. Different letters indicate statistically significant differences ($P < 0.01$)

medium after 48 h of incubation (Fig. 6e), indicating that the plasma membrane integrity of the *ΔBcSEC4* mutant was severely compromised.

Deletion of *BcSEC4* impairs autophagy in *B. cinerea*

Sec4 has been confirmed to participate in autophagosome formation in *S. cerevisiae*. We analyzed the autophagy process by staining with monodansylcadaverine (MDC), an indicator of autophagic activity (Biederbick, 1995). As shown in Fig. 7a, B05.10 displayed strong fluorescence in the cytoplasm and vacuoles under starvation conditions, but only weak fluorescence was observed in the *ΔBcSEC4* mutant under the same conditions. Next, the autophagosomes were observed by transmission electron microscopy. After mycelia were starved for 6 h, autophagosomes of B05.10 accumulated in the vacuoles, whereas the vacuoles of the *ΔBcSEC4* mutant showed normal morphology (Fig. 7b). Therefore, BcSec4 is required for autophagy in *B. cinerea*.

Deletion of *BcSEC4* increases the expression of metabolism-related genes and secretion-related genes

To further study the effect of *BcSEC4* deletion, we performed a comparative transcriptome analysis between B05.10 and the *ΔBcSEC4* mutant strains. We identified 2,104 differentially expressed genes, among which 1258 were up-regulated and 846 were down-regulated (Fig. 8a). Functional annotation of differentially expressed genes was performed using GO and KEGG pathways (Fig. 8b). These genes were enriched in metabolism and ribosome. In the secondary metabolite biosynthetic pathway, genes related to botcinic acid, botrydial, and oxalic acid were upregulated, including *BCIN_12g06390* (botrydial synthesis protein 2-coding gene, *BOT2*), *BCIN_12g06380* (botrydial synthesis protein 1-coding gene, *BOT1*), *BCIN_01g00110* (botcinic acid biosynthesis gene), and others (Fig. 8c). Genes annotated as glycoside hydrolases were also highly enriched, including BCIN_14g03430 (*Polysaccharide Lyase family 1*), BCIN_06g01510 (*glycosyl transferase family 17*), BCIN_01g06010 (*glycoside hydrolase family 16*) (Fig. 8d), and others. Interestingly, conidiation-related genes showed no difference in expression between the *ΔBcSEC4* mutant and B05.10 (Additional file 2: Table S1). These results suggest that BcSec4 is involved in the secretion of enzymes and secondary metabolites.

Discussion

The function of Sec4 is conserved in eukaryotes; it participates in the final step of the exocytic pathway and the fusion of vesicles with the plasma membrane. In yeast (*S.*

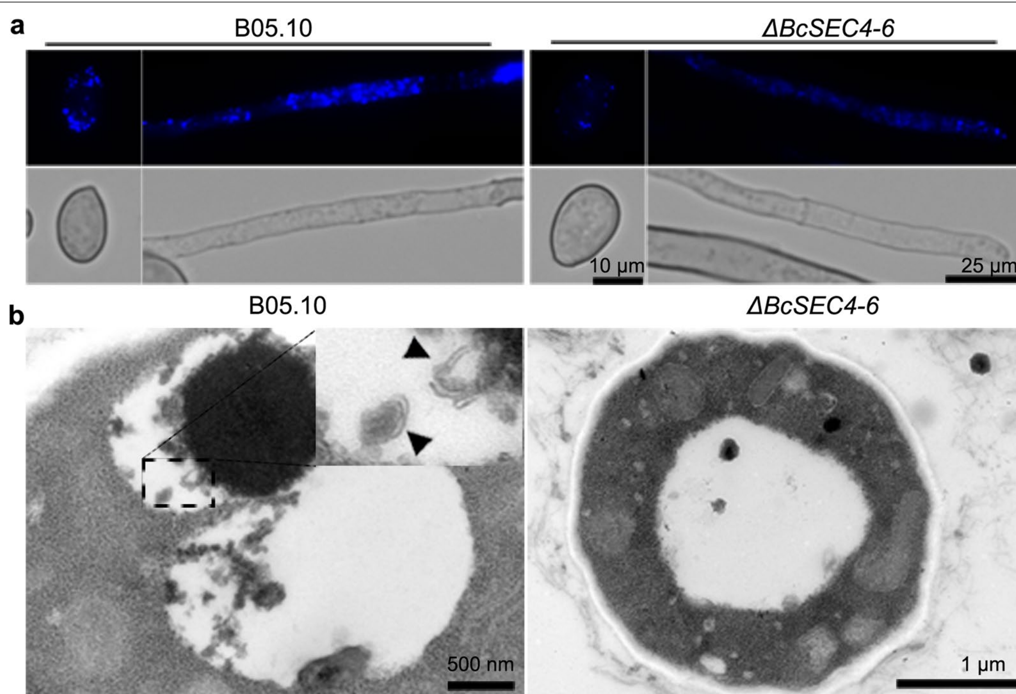

Fig. 7 BcSec4 is involved in autophagy of *B. cinerea*. **a** The starved mycelia and conidia stained with MDC and observed under a fluorescence microscope. **b** Transmission electron microscopy observation of mycelia of B05.10 and the *ΔBcSEC4* mutant cultured in nitrogen-limiting medium (MM-N) with 2 mM PMSF for 6 h

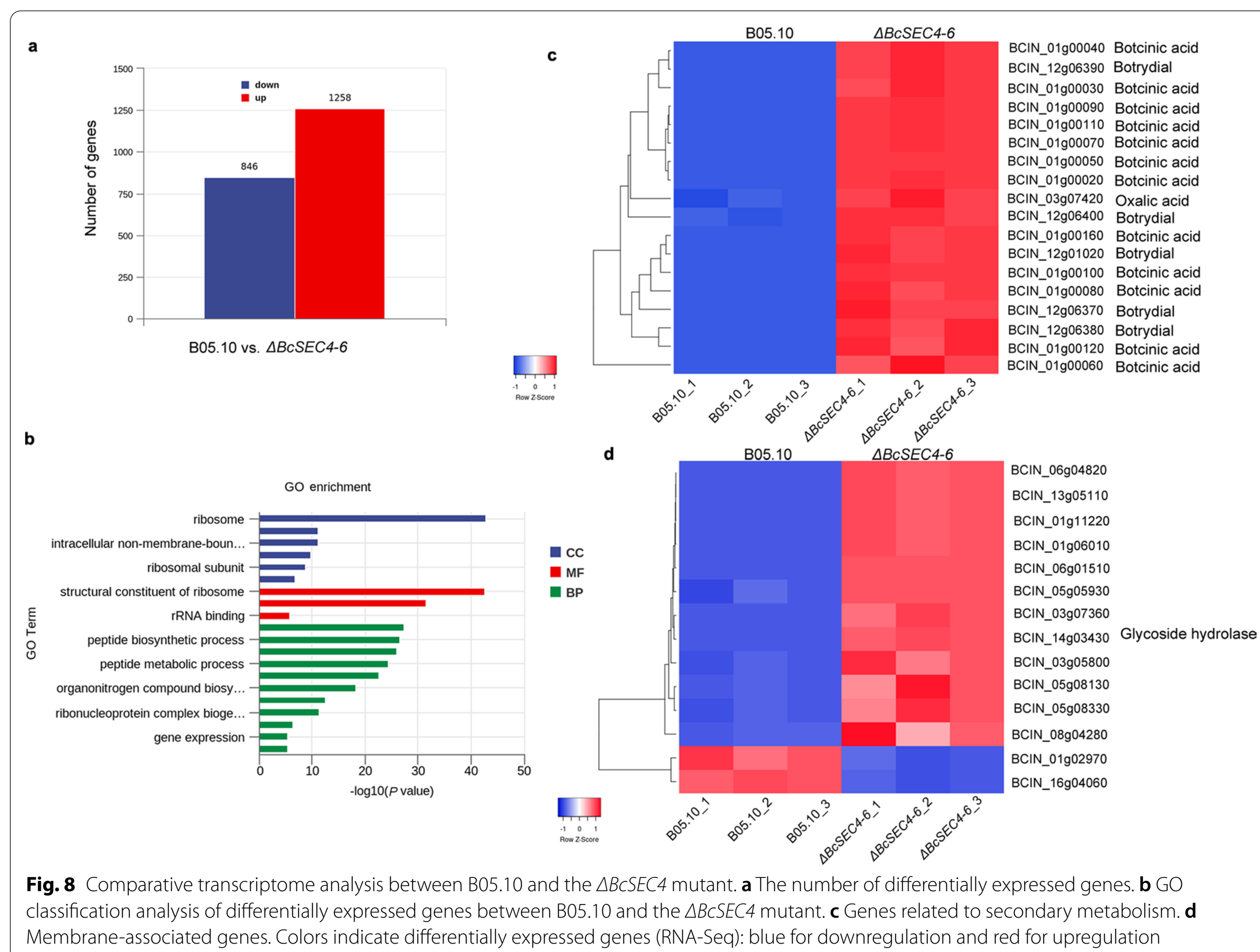

Fig. 8 Comparative transcriptome analysis between B05.10 and the *ΔBcSEC4* mutant. **a** The number of differentially expressed genes. **b** GO classification analysis of differentially expressed genes between B05.10 and the *ΔBcSEC4* mutant. **c** Genes related to secondary metabolism. **d** Membrane-associated genes. Colors indicate differentially expressed genes (RNA-Seq): blue for downregulation and red for upregulation

cerevisiae and *Candida albicans*), Sec4 is essential for viability, and its deletion mutants are nonviable (Salminen and Novick 1987; Mao and Kalb 1999). Unlike yeast, Sec4 is critical for plant pathogenicity in fungi, but it is not essential for viability. In this study, we found that Sec4 affects mycelial growth and conidiophore morphogenesis, as well as responses to various stresses. Sec4 was also critical for the membrane integrity and autophagy.

Similar to its orthologous gene knockout mutants in other plant pathogenic fungi, the *ΔBcSEC4* mutant of *B. cinerea* showed significant reduction in vegetative growth, conidial production, and pathogenicity. The *ΔBcSEC4* mutant had more branches and swollen hyphal tips, similar to previous studies (Zhang et al. 2014b; Zheng et al. 2016; Zhang et al. 2020). Besides Sec4 GTPases, several other GTPases in filamentous phytopathogens have been reported to participate in many important functions, including development and pathogenicity. In *M. oryzae*, MoYpt7, MoRac1, MoCdc42, and MoRho3, a subfamily of the Ras superfamily, were shown to play important roles in the regulation of polarized growth and conidiation (Zheng et al. 2007; Chen et al. 2008; Wu et al. 2009; Liu et al. 2016). Striking defects in producing aerial hyphae were observed in the *ΔMoYPT7* mutant, and it also had defects in conidiophore development and conidium morphology (Liu et al. 2016). The *ΔMoRHO3* mutant formed abortive appressoria (Zheng et al. 2007). In *F. graminearum*, five small GTPases, FgRab6, FgRab7, FgRab8, FgRab51, and FgRab52, were shown to regulate vegetative growth, and their deletion mutants showed drastic reductions in mycelial growth. Microscopic examination revealed that more hyphal branches were formed in the hyphae of *ΔFgRAB6*, *ΔFgRAB8*, and *ΔFgRAB52* mutants compared with wild type (Zheng et al. 2016). These findings indicate that Rab GTPases play important roles in the mycelial morphology and conidiogenesis of filamentous phytopathogens.

Conidial production was significantly decreased in *SEC4* mutants of *M. oryzae*, *F. verticillioides*, and *Aspergillu niger*. Here, we found that the conidiophore morphology of the *ΔBcSEC4* mutant was abnormal compared with that of B05.10, consistent with the result from *A.*

niger. In *A. niger*, GFP-SrgA (the Se4 homolog from *A. fumigatus*) was found to localize to the tips of young conidiophores (Powers-Fletcher et al. 2013). During sporulation in yeast, fluorescence signals for Sec4 were found to accumulate inside of growing membranes. Furthermore, Sec4 temperature-sensitive mutants showed a sporulation-defective phenotype, suggesting that Sec4 is required for prospore membrane formation (Yasuyuki et al. 2017). We therefore suggest that Sec4 does not regulate conidial formation, but presumably maximizes the efficiency of vesicle delivery to the developing condiophore. In the *B. cinerea* transcriptome, the expression of conidiation-associated genes did not differ significantly between B05.10 and the *ΔBcSEC4* mutant. Consequently, although conidial production was significantly decreased, this may have been caused by defects in conidiophore development. These results were further confirmed in *F. verticillioides*: *ΔFvSEC4* null mutation did not impair the expression of key conidiation-related genes (Yan et al. 2019). The *SEC4* mutant also showed a drastic reduction in vegetative growth, but the length of the germ tube at 6 h did not differ significantly between the *ΔBcSEC4* mutant and B05.10. The mycelial growth was clearly affected after 48 h of culture. Consistent with this result, the vegetative growth of the *ΔFgMON1* mutant in *F. graminearum* was significantly reduced, but there was no significant change in the length of germ tubes (Li et al. 2015).

In yeast, the Rab GTPase Sec4 mediates docking and fusion of post-Golgi vesicles and regulates polarized delivery of vesicles to the exocyst at the plasma membrane (Wei et al. 1999). In our results, BcSec4 was required for plasma membrane integrity. We speculate that docking and fusion of vesicles to the plasma membrane cannot be completed due to the deletion of *BcSEC4*. Indeed, *SEC4* mutants fail to form the prospore membrane at restrictive temperatures (Yasuyuki et al. 2017). In our research, hyphae of B05.10 displayed a more punctate distribution in the cytoplasm compared with those of the *SEC4* mutant. Moreover, under starvation conditions, autophagosomes accumulated in the vacuoles of B05.10, whereas few autophagosomes were observed in the vacuoles of the *ΔBcSEC4* mutant. Rab GTPases are required for vesicle–vacuolar fusion during vacuolar biogenesis in fungi. Under starvation conditions, GFP-MoAtg8 in the *ΔMoYPT7* mutant had a punctate distribution in the cytoplasm, whereas GFP-MoAtg8 in wild-type strain moved to the vacuole. The autophagy process was blocked in the *ΔMoYPT7* mutant (Liu et al. 2016). In other fungi, e.g. *A. nidulans*, Rab1 (a homologue of yeast Ypt1) is involved in autophagosome formation (Pinar et al. 2013). However, the *RAB7* deletion mutant was unaffected in autophagosome formation, although fusion between autophagosomes and vacuoles was affected; FgRab7 therefore regulates the fusion of vacuoles and autophagosomes (Zheng et al. 2018).

Previous experiments have shown that knockout of *BcSEC4* changes the production of laccase and cerato-platanin. Overexpression of the laccase gene *GhLac1* in cotton (*Gossypium hirsutum*) increases tolerance to the fungal pathogen *Verticillium dahlia* and alters JA and JA-Ile contents (Hu et al. 2018). When *EuLAC1*, a laccase gene, was cloned from *Eucommia ulmoides* and introduced into tobacco (*Nicotiana tabacum*), the transgenic tobacco displayed resistance to *B. cinerea* (Zhao et al. 2022). Cerato-platanin from *B. cinerea* and *Sclerotinia sclerotiorum* is an elicitor that triggers a local hypersensitive response in plant leaves and improves plant pathogen resistance. It also plays an important role in virulence (Frías and Brito 2011; Yang et al. 2018). Interestingly, loss of *BcSEC4* in *B. cinerea* induced the expression of JA related genes and reduced pathogenicity. We speculate that the pathogenicity defect of the *ΔBcSEC4* mutant was caused by changes in vegetative growth, secretion of extracellular proteins and up-regulation of JA related genes in the plant.

Conclusions

Our study demonstrates that BcSec4 plays important roles in *B. cinerea*, affecting mycelial growth, plasma membrane integrity, conidiophore morphology, autophagy, and plant infection, although we cannot rule out the possibility that the loss of pathogenicity may be caused by a defect in autophagy or plasma membrane integrity. Future studies will examine the relationship between plasma membrane integrity and pathogenicity in *B. cinerea*.

Methods

Fungal strains and manipulations

The *B. cinerea* strain B05.10 was used as the wild-type strain. All strains were incubated on potato dextrose agar (PDA) medium and cultured at 25 °C as described previously (Liu et al. 2018). Yeast extract peptone dextrose (YEPD) liquid medium was used for collection of mycelia and extracellular proteins. Schenk and Hildebrandt medium (SH) was used for cell wall regeneration of protoplasts (Braun and Heisler 1990). Cell wall integrity was assessed under different stress conditions containing 100 mg/L Congo red, 5 mmol/L H_2O_2, 0.01% SDS, and the diameters of all strains were measured after 4 days of incubation on PDA as described previously (Yin et al. 2018). All assays in this study were performed in three independent biological experiments with at least three replicates.

Targeted gene deletion and complementation of *BcSEC4*

To construct the *BcSEC4* replacement vector, an upstream fragment and downstream fragment of *BcSEC4* and the hygromycin B phosphotransferase cassette (HPH) were amplified from the *B. cinerea* genome and the Pxeh vector, respectively. Then the replacement vector was constructed by fusion PCR and transformed into the B05.10 strain as described previously. The transformants were screened on PDA with 100 μg/mL hygromycin. The deletion mutants were confirmed by PCR. The complementation vector was constructed using a 1.6-kb genomic DNA fragment containing the native promoter, CDS, and 3′ -UTR of *BcSec4*.

Extracellular protein detection

Laccase activity on solid medium was measured as described by Zhang (Zhang et al. 2014a). B05.10 and the *ΔBcSEC4* mutants were inoculated on PDA with the laccase substrate 2, 2'-azino-di-3-ethylbenzathiazoline-6-sulfonate (ABTS, Sigma).

Equal volume of conidial suspensions of B05.10 and the *ΔBcSEC4* mutant were inoculated into 10% YEPD liquid medium for 1 day, and extracellular proteins were precipitated using acetone at 4 °C. The samples were digested, and identification of proteins was performed using a NanoLC-ESI-MS/MS system; the analysis was carried out by ProtTech (Suzhou, China) (Massa et al. 2019).

Plant infection assay

Conidial suspensions (2×10^5 conidia/mL in 10% YEPD solution) were harvested from the tested *B. cinerea* strains cultured on PDA plates for 7–10 days. The leaves of mung bean and tomato were used for pathogenicity assays. Droplets (10 μL) of conidial suspension were inoculated onto detached leaves of mung bean and tomato. Inoculated leaves were incubated in chambers at 25 °C for 72 h under high humidity conditions. Lesion diameters on leaves were examined, and photographs were taken at 3 dpi.

Fungal penetration assay

For penetration assays, conidial suspensions (2×10^5 conidia/mL) of the tested *B. cinerea* strains were inoculated onto onion epidermal layers and incubated at 25 °C for 12 h under humidity conditions. Before observation under a light microscopy, samples were stained with lactophenol blue as described previously (Zhang et al. 2020). Hyphae staining indicated that hyphae were on the surface of the onion, but infection hyphae were not stained by lactophenol blue. The infected samples were observed under the microscope at 12 and 36 hpi.

Staining and ultrastructure observation

Conidial suspensions of each strain were collected and inoculated into YEPD liquid medium, then cultured for 12 h at 25 °C. Germ tubes were collected and stained with propidium iodide (PI) (Beyotime, Shanghai, China) and DCFH-DA (Beyotime, Shanghai, China) according to manufacturer's instructions. The samples were then observed under a microscope (Olympus BX60).

For transmission electron microscope (TEM) observation, the germ tubes (conidia from the *ΔBcSEC4* mutant or B05.10 were incubated in YEPD for 12 h, washed thoroughly with distilled water, and transferred to MM-N liquid medium with 2 mM phenylmethylsulfonyl fluoride [PMSF], then incubated for another 6 h) were harvested and fixed with 2.5% glutaraldehyde for 1.5 h, then washed for 15 min three times with 0.1 M phosphate buffer. After fixation, the samples were dehydrated in a graded ethanol series (once at 30, 50, 70, 80, and 90% and twice at 100%) for 15 min at each concentration. Finally, they were embedded in resin and stained with 2% uranyl acetate and citrate. The samples were examined under a JEM-1230 electron microscope (JEOL, Japan).

Electrical conductivity test

B. cinerea was inoculated into potato dextrose broth (PDB) medium and incubated at 25 °C and 160 rpm for 2 days. The electrical conductivity of the supernatant was then determined to assess changes in the release of cellular materials. The electrical conductivity and absorbance at 260 nm of the obtained supernatants were measured with a conductivity meter (DDS-11A, Shanghai Precision Scientific Instrument Co., Ltd. Shanghai, China) (Shao et al. 2013).

RNA preparation and reverse transcription-quantitative PCR (RT-qPCR) analysis

Leaves of two-month-old plants were inoculated with conidial suspension (5.4×10^4 conidia/mL) for 12 h. Samples were then collected, and total RNA was extracted using the TRIzol reagent. Reverse transcription of total RNA was carried out using a PrimeScript RT Regent Kit with gDNA Eraser (Takara, Japan). Quantitative real-time PCR (qPCR) analyses were performed using TB Green® Premix EX *Taq* (TaKaRa, Japan) to analyze the relative expression level of plant defense-related genes (Zhang et al. 2014a). Expression levels were normalized with the actin gene as an internal control.

Transcriptome sequencing

B05.10 and the *ΔBcSEC4* mutant were cultured in YEPD medium at 25 °C for 2 days. Total RNA was extracted with the TRIzol reagent, and RNA quality was assessed on 1% agarose gels. High-throughput RNA-seq was

performed on the Illumina HiSeq 2000 platform (Shanghai Personal Biotechnology Co., Ltd, China) to obtain 200-bp paired-end reads. The reads were mapped to the *B. cinerea* reference genome (https://mycocosm.jgi.doe.gov/pages/blast-query.jsf?db=Botci1) using TopHat and bowtie2 (Trapnell et al. 2010).

Genes differentially expressed between the *ΔBcSEC4* mutant and B05.10 were identified using DESeq2. Genes with a log2 Fold Change (FC) > 1 and adjusted *P*-value < 0.05 were considered to be differentially expressed. Functional annotation of differentially expressed genes was performed based on Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis with clusterProfiler (3.4.4). Each genotype was replicated three times.

Abbreviations

ABTS: 2, 2′-Azino-di-3-ethylbenzathiazoline-6-sulfonate; CR: Congo red; DCFH-DA: Dichlorofluorescein diacetate; FC: Fold change; GO: Gene ontology; HPH: Hygromycin B phosphotransferase cassette; KEGG: Kyoto Encyclopedia of Genes and Genomes; MDC: Monodansylcadaverine; PI: Propidium iodide; PMSF: Phenylmethylsulfonyl fluoride; PDA: Potato dextrose agar; SDS: Sodium dodecyl sulfate; TEM: Transmission electron microscope; YEPD: Yeast extract peptone dextrose.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-022-00131-3.

Additional file 1: **Figure S1.** BcSec4 is indispensable for vegetative growth of *B. cinerea*. **a** Colony morphology of B05.10, the *ΔBcSEC4* mutants, and complemented strains grown on PDA plates for 4 days. **b** The *ΔBcSEC4* mutant displayed enhanced hyphal branching compared to B05.10 after incubation on PDA plates for 3 days. **c** Morphology of hyphal tips of B05.10 and the *ΔBcSEC4* mutant growing on PDA plates for 3 days. **Figure S2.** BcSec4 is involved in protein secretion. **a** The hypersensitive response in *Nicotiana benthamiana* elicited by the supernatants of B05.10 and the *ΔBcSEC4* mutant. The supernatants were collected after incubation in YEPD liquid medium for 24 h. **b** SDS-PAGE analysis of extracellular proteins produced by B05.10 and the *ΔBcSEC4* mutant. **c** Functional classification of extracellular proteins produced by B05.10 and the *ΔBcSEC4* mutant. **Figure S3.** BcSec4 is required for full virulence of *B. cinerea*. Tomato (**a**) and mung bean (**b**) leaves were inoculated with mycelial plugs at 25 °C in the dark. **Figure S4.** Disruption of BcSEC4 did not impair infection cushion formation. **a** Infection cushion formation of B05.10 and the *ΔBcSEC4* mutant on a glass surface at 24 hpi. **b** Quantification of infection cushion formation by B05.10 and the *ΔBcSEC4* mutant. **Figure S5.** The expression levels of JA-related genes determined via RT-qPCR. Levels of transcripts were normalized against that of *β-actin*. Three biological replicates were performed independently. Data are represented as means ± standard deviations (SDs) from three independent experiments

Additional file 2. Table S1. Conidiation-associated genes in *B. cinerea*

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Author contributions

DL and GW conceived the experiments. GW, YW, and JZ performed the experiments. DL, GW, YW, JZ, and WL analyzed and interpreted the data. DL supervised the work and wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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