Knocking Out *Bcsas1* in *Botrytis cinerea* Impacts Growth, Development, and Secretion of Extracellular Proteins, Which Decreases Virulence

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Pathogenic fungi usually secrete a series of virulence factors to the extracellular environment to facilitate infection. Rab GTPases play a central role in the secretory pathway. To explore the function of Rab/GTPase in filamentous fungi, we knocked out a Rab/GTPase family gene, *Bcsas1*, in *Botrytis cinerea*, an aggressive fungal pathogen that infects more than 200 plant species. A detailed analysis was conducted on the virulence and the secretory capability of the mutants. The results indicated that knockout of *Bcsas1* inhibited hyphal development and reduced sporulation of *B. cinerea* on potato dextrose agar plates resulting in reduced virulence on various fruit hosts. Knocking out the *Bcsas1* gene led to an accumulation of transport vesicles at the hyphal tip, significantly reduced extracellular protein content, and lowered the activity of polygalacturonase and xylanase in the extracellular medium. However, mutation of *Bcsas1* did not affect the expression of genes encoding polygalacturonase and xylanase, suggesting the secretion of these two family enzymes was suppressed in the mutant. Moreover, a comparative analysis of the secretome provided further evidence that the disruption of *Bcsas1* in mutant strains significantly depressed the secretion of polysaccharide hydrolases and proteases. The results indicate that *Bcsas1*, the *Rab8/SEC4*-like gene, plays a crucial role in development, protein secretion, and virulence of *B. cinerea*.

Botrytis cinerea, a necrotrophic fungal phytopathogen, causes gray mold rot in more than 200 host plant species and is especially destructive on fruits and vegetables, causing serious economic losses (Williamson et al. 2007). *B. cinerea* can secrete an arsenal of enzymes and metabolites into the extracellular environment during its infection process. Some of them, including oxalic acid (Kim et al. 2008), Nep1-like proteins (Schouten et al. 2008), cerato-platanin family protein (Frías et al. 2011), and xylanase (Noda et al. 2010) have the ability to induce host responses characteristic of a hypersensitive response. In addition, other cell wall-degrading enzymes

secreted by this phytopathogen during early stages of infection, such as endopolygalacturonase (ten Have et al. 1998), pectin methylesterase (Valette-Collet et al. 2003), and xylanase (Brito et al. 2006), have been reported to be necessary for successful establishment and proliferation (lesion extension) in plant tissues. Extracellular proteins also play a role in the molecular dialogue associated with host-pathogen interactions (Esquerré-Tugayé et al. 2000), suggesting that a precise regulation of these proteins should exist during pathogenesis. Protein secretion by filamentous fungi usually occurs at the hyphal tip or subapical hyphal regions (Conesa et al. 2001). Secretory proteins begin their transport to the extracellular environment by entering the endoplasmic reticulum, in which they are properly folded and modified (Sakaguchi 1997). The proteins are then packed in transport vesicles and transported to the Golgi compartment in which further modifications take place (Novick and Zerial 1997). Finally, the mature proteins are transported to the plasma membrane by secretory vesicles that fuse with the plasma membrane, allowing the secretory proteins to be released to the extracellular environment (Conesa et al. 2001). Many studies on vesicle transport in mammalian cells and yeast have been reported (Chanda et al. 2009; Chen et al. 2001; Van den Hazel et al. 1996; Walworth et al. 1989), but little information is available for filamentous fungi.

As the largest branch of the Ras superfamily, Rab proteins constitute a family of small GTPases that are required for vesicle docking and fusion and, thus, play a central role in the secretory pathway (Novick and Zerial 1997; Punt et al. 2001). Small GTPases function by cycling between active (membrane-associated) and inactive (soluble) GTP-bound states. Some protein effectors are involved in the regulation of Rab activity during this cycling, such as a GDP/GTP exchange factor, which catalyzes GDP/GTP conversion, and GTPase-activating proteins that accelerate GTP hydrolysis. Each GTPase has a specific subcellular localization and takes part in a specific stage of the secretory pathway (Novick and Zerial 1997). Rab family proteins have a number of conservative domains, including four GTP-interaction domains (G1 to G4), five Rab-specific functional domains (F1 to F5), and four subfamily-specific domains (SF1 to SF4) (Pereira-Leal and Seabra 2000; Stenmark and Olkkonen 2001).

Arabidopsis and mammals each have roughly 60 Rab GTPases (Pereira-Leal and Seabra 2001; Rutherford and Moore 2002). The model, unicellular organism *Saccharomyces cerevisiae* has 11 Rab family proteins, named as YPT or SEC4 (Pereira-Leal and Seabra 2001). The number of Rab proteins ranges from eight to 12 in most of the sequenced fungi (Pereira-Leal 2008). Although few genes involved in vesicle secretion in filamentous fungi have been cloned, sequence information from

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these clones can be used to search for homologues that contribute to vesicle secretion in other organisms. In the present work, we isolated and characterized a Rab family gene, *Bcsas1*, from *B. cinerea*. *Bcsas1* is a homologue of *Rab8* and *SEC4*. *SEC4* was the first Rab protein identified in yeast and was demonstrated to play an important role in both growth and protein secretion (Clément et al. 1998; Mao et al. 1999; Salminen and Novick 1987). There have been reports on two *SEC4*-like Rab genes (*srgA* and *CLPT1*) from filamentous fungi. In *Aspergillus niger*, disruption of *srgA* led to reduced protein secretion and unusual apical branching but was not essential for growth (Punt et al. 2001). In contrast, disruption of *CLPT1* in *Colletotrichum lindemuthianum* resulted in a lethal phenotype (Dumas et al. 2001). *CLPT1* gene function was further studied using a mutant with a dominant-negative allele, and it was ascertained that *CLPT1* was necessary for fungal pathogenesis, because it regulated the intracellular transport of secretory vesicles involved in the delivery of proteins to the extracellular medium (Dumas et al. 2001) and the differentiation of infectious structures (Siriputthaiwan et al. 2005). Although the previous reports suggest that the function of *SEC4*-like genes is conserved in different organisms, some variability has been noted. In order to explore the function of Rab genes in filamentous fungi, we disrupted a Rab/GTPase family gene (*Bcsas1*) in *B. cinerea* using homologous recombination and conducted a detailed analysis of the virulence and secretory capability of the resulting mutants. The results indicate that *Bcsas1* plays a crucial role in development, protein secretion, and virulence of *B. cinerea*.

RESULTS

Bcsas1 encodes a Rab GTPase with homology to Rab8/SEC4.

A secretion-related Rab/GTPase gene, *Bcsas1* (BC1G_14039.1), was identified using the *B. cinerea* B05.10 genome database. The *Bcsas1* genomic sequence consists of 989 bp,

and the coding sequence is interrupted by two introns. The deduced *Bcsas1* protein is composed of 203 amino acids with an estimated molecular mass of 22.52 kDa and an isoelectric point (pI) of 7.09. *Bcsas1* encodes a Rab GTPase with greatest homology to *Rab8* and *SEC4*, both of which have been shown to be involved in post-Golgi transport to the plasma membrane in mammals and yeasts (Chen et al. 2001; Goud et al. 1988; Huber et al. 1993; Peranen et al. 1996; Walworth et al. 1989). Alignment of amino acid sequences showed the sequence of *Bcsas1* protein with the specific hits of *Rab8/SEC4* and 54.19% identity with *SEC4* protein of *S. cerevisiae*, 58.37% with *Rab8* protein of *Drosophila melanogaster*, and 60.59% with *Rab8* protein of *Mus musculus* (Supplementary Fig. S1). Phylogenetic tree analysis also showed that *Bcsas1* protein has significant similarity to *SEC4* protein of *S. cerevisiae* (Supplementary Fig. S4).

The expression of *Bcsas1* was induced by oxidative stress.

Rapid accumulation of reactive oxygen species, referred to as an oxidative burst, often occurs in the host at the site of pathogen invasion and is considered an early defense reaction (Segal 2005; Temme and Tudzynski 2009). Preliminary experiments indicated that exogenous application of 5.0 mM H_2O_2 could inhibit germination of conidia by 80%, and at 10 mM, germination was inhibited completely (Supplementary Fig. S2). Quantitative real-time polymerase chain reaction (PCR) was carried out to determine if oxidative stress could affect the expression pattern of *Bcsas1* in *B. cinerea*. The conidia were incubated in potato dextrose broth (PDB) till germinated (8 h), and then, H_2O_2 was added before further incubation for 1 h. The result indicated that the expression of *Bcsas1* was significantly upregulated under H_2O_2 stress (Fig. 1). The average relative transcript abundance of *Bcsas1* was 118% higher than the control in response to 10 mM H_2O_2 (Fig. 1). There are 10 Rab family genes in *B. cinerea*. The results showed that all these genes were upregulated upon exposure to H_2O_2 stress (Fig. 1),

Fig. 1. Expression patterns of Rab family genes in *Botrytis cinerea* under oxidative stress. The conidia of the wild-type B05.10 were incubated in potato dextrose broth for 8 h, and the RNA was extracted after 1-h treatment with H_2O_2 . *Bcactin* (BC1G_08198.1) and *Bctubulin* (BC1G_00122.1) were used as loading controls. Bars indicate standard deviation of means of three replications.

suggesting that Rab family genes in *B. cinerea* could respond to oxidative stress.

The generation of *Bcsas1* deletion mutants.

Bcsas1 deletion mutants were obtained using a gene replacement strategy (Fig. 2A). A part of the gene sequence (158 bp) was replaced with a hygromycin-resistance cassette. The deduced amino acids coded by the deleted DNA sequence contains five conserved domains, including a putative GDI interaction site, putative effector interaction site, Rab family motif 4, Rab family motif 5, and switch II region. A total of 40 independent hygromycin-resistant colonies were obtained from one transformation experiment. Flank-spanning PCR was used to verify the insertion in the colonies, using SJ-F forward and SJ-R reverse primers, which are located outside the L flank and inside the hygromycin-resistance cassette, respectively (Fig. 2A). A 2.4-kb fragment was amplified only from the transformants, which indicated the correct integration. Three of the hygromycin-resistant colonies, $\Delta bs3$, $\Delta bs5$, and $\Delta bs8$, showed the diagnostic fragment (Fig. 2B). To exclude heterokaryons, one round of single-spore isolation was carried out. Three homokaryotic *Bcsas1* deletion mutants, named as $\Delta bs3-3$, $\Delta bs5-11$, and $\Delta bs8-13$, were obtained. The PCR assay of an internal fragment showed that *Bcsas1* was completely removed from the mutants (Fig. 2C). The three mutants were subjected to Southern blot analysis, using the labeled L fragment as a probe to ensure that there was

no ectopic integration. The wild-type strain B05.10 showed a 2.6-kb hybridizing band and the three mutants had a 5.0-kb band, as expected (Fig. 2D). These results indicated that the three mutants had a single copy insertion of *Bcsas1*.

Bcsas1 mutants exhibit reduced radial growth, sclerotia formation, and sporulation.

Dramatic changes in vegetative growth were observed in the *Bcsas1* mutants. Radial growth was evaluated as colony diameter on potato dextrose agar (PDA) plates. The growth rate of mutants was drastically reduced compared with wild-type strain B05.10 (Fig. 3A and C). On the fourth day after inoculation, the colony diameter of the wild type strain was as large as the plate (90 mm). In contrast, the diameter of the mutant strains was only about 10 mm on average (Fig. 3A). Mycelia of mutants were thinner, with more branches as compared with the wild-type strain. Hyphal tips of the mutants frequently appeared swollen (Fig. 3B, arrows). The three *Bcsas1* mutants did not develop sclerotia as was observed in the wild-type B05.10 (Fig. 3A). The mutant $\Delta bs3-3$ and wild-type strain were cultured on PDA plates amended with different concentrations of hydrogen peroxide in order to determine the effect of the *Bcsas1* deletion on growth of *B. cinerea* under oxidative stress. The mutant showed a slight reduction in growth compared with wild type, implying that the mutant is less sensitive to oxidative stress than wild type (Fig. 3D). The quantity of

Fig. 2. Generation of *Bcsas1* mutants. **A**, Replacement strategy for deletion of *Bcsas1*. Flank L and Flank R were amplified by primer pairs BSL-F and BSL-R and BSR-F and BSR-R, respectively. The replacement vector pABS was constructed by cloning Flank L and Flank R on each side of the hygromycin-resistance cassette of pLOB7. **B**, Detection of the mutants by flank-spanning polymerase chain reaction (PCR). A 2.4-kb fragment amplified with primer pair SJ-F and SJ-R from the transformants deriving from homologous integration. **C**, PCR assay of the internal fragment of *Bcsas1* in wild type and mutants. **D**, Southern blot analysis of B05.10 (WT) and three mutants, $\Delta bs3-3$, $\Delta bs5-11$, and $\Delta bs8-13$. The genomic DNA was digested with *EcoRV*, separated in agarose gel, blotted, and hybridized with probe (Flank L labeled with digoxigenin). Primer binding sites are indicated by arrows and probe is indicated by black bar.

conidia was assessed 7 days postinoculation. Production of conidia in the wild-type strain was more than 100-fold higher than in the mutant strains (Fig. 4A and B). In sharp contrast, the rate of germination and mycelial growth in the wild type and mutants did not differ when they were grown in liquid culture. After 8 h of incubation, almost all the conidia had germinated (Fig. 4C). The dry weight of mycelium did not show a significant difference between wild type and mutants after culturing for 3 days in Czapeck liquid medium (Fig. 4D). Complementation of the mutant $\Delta bs3-3$ with the *Bcsas1* gene under control of its own promoter and terminator restored wild-type growth and colony morphology (Figs. 3 and 4).

***Bcsas1* mutants exhibit less virulence on host plants.**

To determine the virulence of the mutants on host plant tissue, we first monitored the penetration process on onion epidermis and found that the *Bcsas1* mutants could successfully penetrate the epidermis and develop in epidermal cells (Supplementary Fig. S3). Tomato and apple fruit as well as tomato leaves were then selected as hosts to further test the virulence of *Bcsas1* mutants. On tomato fruit, lesion expansion rates of the three mutants were significantly lower than that of wild-type strain B05.10. In contrast to the mutants, the B05.10 strain also developed more mycelia and sporulated abundantly at the wound site (Fig. 5A and D). On apple fruit, the virulence of *Bcsas1* mutants was sharply depressed. After 4 days, slight lesions

were detected in the mutant-inoculated apple fruit, whereas lesion size in B05.10-inoculated fruit was 30 mm (Fig. 5B and E). However, mutant strains could not penetrate the epidermis of detached tomato leaves (Fig. 5C and F). Complementation of the mutant strain, however, could restore its virulence, making it similar to the wild-type strain. The results demonstrated that the virulence of the *Bcsas1* mutants was significantly impaired on different plant hosts.

Disruption of *Bcsas1* blocks protein secretion.

To determine if the disruption of *Bcsas1* affects exocytosis, mycelia of B05.10 and the *Bcsas1* mutant $\Delta bs3-3$ were stained with FM4-64, an amphiphilic styryl dye that can insert itself into the outer leaflet of the plasma membrane. This dye has been widely used as a fluorescent reporter of components of the vesicle trafficking network (Fischer-Parton et al. 2000; Vida and Emr 1995). A stronger fluorescent signal, indicating the accumulation of trafficking vesicles, was detected at the hyphal tips of the mutant strains, compared with B05.10 (Fig. 6A). In order to analyze the polysaccharide in the cell walls of the wild-type strain and mutant, Calcofluor white staining was performed. The result showed that the distribution of polysaccharides had stronger polarity at the tip part of the wild-type strain, compared with the mutant (Fig. 6B). This implied that cell-wall synthesis is more active at the hyphal tip of the wild type than that of the mutant. To test the possibility that *Bcsas1*

Fig. 3. Radial growth and mycelial morphology of the wild type and mutants on potato dextrose agar (PDA) plates. **A**, Colony phenotype of B05.10, the mutants, and the complemented strain after growing for 7 days on PDA plates. **B**, Morphology of 3-day-old hyphae growing on PDA plates. The scale bars represent 20 μ m. **C**, Radial growth rate on PDA plates. Bars indicate standard deviation of means of three replications. **D**, Colony diameters of mutant $\Delta bs3-3$ and B05.10 after 3 days growth under H_2O_2 stress.

Fig. 4. The sporulation, conidia germination, and mycelial dry weight assays. **A**, Sporulation examination of wild type, mutants, and the complemented strain, incubated for 7 days, under microscope. Arrows indicate the conidia. Scale bars represent 50 μ m. **B**, Conidium production of wild type, mutants, and the complemented strain, incubated for 7 days. **C**, Germination rate examination of wild type, mutants, and the complemented strain in potato dextrose broth. **D**, The dry weight of mycelium assay. A suspension of 5×10^6 conidia was inoculated into 100 ml Czapeck liquid medium and was cultured for 3 days.

Fig. 5. **A**, Virulence assays on tomato fruit, 3 days after inoculation, **B**, apple fruit, 4 days after inoculation, and **C**, on detached tomato leaves, 2 days after inoculation. **D**, Statistical analysis of the lesions on tomato fruits, **E**, on apple fruits, and **F**, on detached tomato leaves. The tomato and apple fruits were wounded with a sterile nail, and the leaves were wounded slightly with a hairbrush before inoculation.

regulates protein secretion, proteins were extracted from the supernatant of cultures growing in Czapeck liquid medium for 3 days. Proteins were separated by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Results indicated that the quantity of extracellular protein in the supernatant obtained from mutant cultures was significantly lower than in supernatant extracts obtained from wild-type cultures (Fig. 6B). The disruption of the *Bcsas1* gene also led to a dramatic reduction in polygalacturonase and xylanase activity (Fig. 6C), both of which are crucial pathogenic factors secreted by *B. cinerea* (Brito et al. 2006; ten Have et al. 1998). In contrast, there was no significant difference, at the transcription level, of the polygalacturonase and xylanase genes between wild type and mutants (Fig. 6E). The results indicated that the secretion of these two family extracellular hydrolytic enzymes was suppressed in mutant.

Analysis of the secretome.

Comparative analysis of the wild-type B05.10 and *Bcsas1*-deletion mutant secretome was performed. To obtain sufficient extracellular proteins, strains were cultured in Czapeck liquid medium for 120 h, and proteins were extracted from the supernatant and were subjected to two-dimensional (2-D) electrophoresis. First-dimension separation utilized immobilized pH gradient strips spanning a pH range of 4 to 7. More than 400 protein spots were detected in each gel, using Image Master 2-D Elite software (Fig. 7A). Spots that showed statistically significant changes ($P < 0.05$) of more than 1.5-fold in relative abundance were designated as differential spots. A total of 33 differentially expressed proteins were detected in the secretome of *Bcsas1* deletion mutant, including 24 downregulated and

nine upregulated proteins. Ignoring very faint spots and spots with undefined shapes and areas, the remaining 24 spots were analyzed by tandem mass spectrometry (MS/MS). Using the NCBI database, 19 of the 24 spots were identified with MOWSE scores significantly higher than the threshold ($P < 0.05$). These included 14 downregulated proteins and five up-regulated proteins (Fig. 7B). Protein spots D8 and D9 (alpha-L-arabinofuranosidase) and spots U1, U2, and U3 (immunoglobulin E-binding protein) were identified as the same protein. Their location in the gels (as defined by their pI and molecular mass) differed slightly, perhaps due to different posttranslational modifications of the same protein. The group of down-regulated proteins was composed of nine polysaccharide hydrolases, four proteases, and one lipase (Fig. 7C). The putative subcellular localization of the identified proteins was identified using the SignalP algorithm with ProtComp 9.0. Most of the identified proteins have designations of extracellular localization, except N2 (cytoplasm) and U9 (mitochondria) (Supplementary Table S2). The appearance of N2 (glutamate carboxypeptidase) and U9 (mannitol dehydrogenase) in the secretome may have been the result of cell lysis or secretion through a nonclassical secretory pathway.

DISCUSSION

Various mechanisms exist that allow plant hosts and microbes to establish a “molecular dialogue” that directly impacts virulence and pathogenicity (Esquerré-Tugayé et al. 2000). Pathogenic fungi often secrete a series of virulence factors into the extracellular environment that facilitate infection and allow the pathogen to assimilate essential nutrients from the host (Conesa

Fig. 6. Determination of secretion ability. **A**, Confocal image of growing hyphal tip of wild-type B05.10 and mutant $\Delta bs3-3$ stained with 5 μ M FM4-64. The scale bars represent 10 μ m. **B**, Calcofluor white staining at hyphal tip of B05.10 and $\Delta bs3-3$. **C**, Secreted proteins separated by 12% Tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver stained. The loaded proteins were extracted from the same fungal biomass determined as dry weight. **D**, The activities of cell wall-degrading enzymes in the culture supernatants of wild-type B05.10 and mutant $\Delta bs3-3$ incubated for 3 days. Bars indicate standard deviation of means of three replications. **E**, Expression assays of polygalacturonase genes and xylanase genes in wild-type B05.10 and $\Delta bs3-3$ incubated for 3 days. Gene ID: P1, BC1G_00240.1; P2, BC1G_13367.1; P3, BC1G_04246.1; P4, BC1G_02003.1; P5, BC1G_00230.1; P6, BC1G_08033.1; XA, BC1G_00576.1; XB, BC1G_03590.1; and XC, BC1G_01778.1. Bars indicate standard deviation of means of three replications.

et al. 2001; Espino et al. 2010). Extracellular secretion is dependent on vesicle transport and Rab GTPases are well-established regulators of this process (Novick and Zerial 1997). Rab GTPases have been shown to influence major steps in vesicle transport, such as vesicle budding, delivery, tethering, and fusion of the vesicle membrane with the target compartment (Grosshans et al. 2006). Based on a genomic analysis of *B. cinerea*, we identified 10 Rab family genes (Table 1). Expression of the 10 Rab genes was upregulated in response to exogenous oxidative stress (Fig. 1), suggesting that they are involved in plant-microbe interactions.

The homologues of *Bcsas1*, *Rab8* and *SEC4*, have been extensively studied in model organisms (Huber et al. 1993; Peranen et al. 1996) and have been reported to be involved in post-Golgi transport of vesicles to the plasma membrane (Nielsen et al. 2008). *Rab8* in mammalian cells and *SEC4* in yeast are essential to several life processes (Goud et al. 1988; Hattula et al. 2006; Salminen and Novick 1987). *Rab8*-deficient mice exhibit a marked reduction in the absorption rate of nutrients in the small intestine, which eventually leads to death (Sato et al. 2007). In *S. cerevisiae*, *SEC4* null mutants are non-viable (Mao et al. 1999). The dominant-negative mutations generated by substituting different amino acids coded by the *SEC4* gene inhibited growth and protein secretion (Walworth et al. 1989). In contrast to *SEC4*, disruption of *Bcsas1* in *B. cinerea* was not lethal but had a striking effect on hyphal growth and morphology on solid medium (Fig. 3). The mutants were characterized by smaller, compact colonies as well as reduced sporulation on PDA (Fig. 4). Punt and associates (2001) re-

ported that disruption of *srgA*, a homologue of *SEC4*, in *Aspergillus niger*, also led to a slower growth rate. They suggested the effect on hyphal growth was due to the disruption of the proper targeting of secretory vesicles containing cell-wall synthases and hydrolases to the plasma membrane (Punt et al. 2001). The expression of *Bcsas1* was induced under H₂O₂ stress, suggesting that this gene plays an important role in response to H₂O₂ stress. Interestingly, we found that *Bcsas1* mutants were less sensitive to oxidative stress compared with wild type (Fig. 3D). This implies that *Bcsas1* might negatively influence the response of *B. cinerea* to H₂O₂ stress. To better understand the function of *Bcsas1*, we stained hyphal tips of *B. cinerea* with FM4-64 and found the highest level of fluorescence at the hyphal tips of mutant strains, indicating that vesi-

Table 1. The Rab family genes of *Botrytis cinerea* according to alignment of the protein sequence at the National Center for Biotechnology Information database

Gene name	Gene ID	Orthologous gene
<i>Bcsas1</i>	BC1G_14039.1	<i>Rab8, Sec4</i>
<i>Bcypt1a</i>	BC1G_02688.1	<i>Ypt1, Rab1</i>
<i>Bcypt1b</i>	BC1G_09346.1	<i>Rab11</i>
<i>Bcyptv3</i>	BC1G_06093.1	<i>Rab18</i>
<i>Bcrab6</i>	BC1G_08710.1	<i>Rab6</i>
<i>Bcrab7</i>	BC1G_07591.1	<i>Rab7</i>
<i>Bcypt52a</i>	BC1G_05919.1	<i>Ypt52, Rab5, Rab22</i>
<i>Bcypt52b</i>	BC1G_07328.1	<i>Ypt52, Rab5, Rab22</i>
<i>Bcypt4a</i>	BC1G_01676.1	<i>Rab4</i>
<i>Bcypt4b</i>	BC1G_12412.1	<i>Rab2</i>

Fig. 7. Two-dimensional (2-D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of secretome. **A**, 2-D electrophoresis patterns of secretome from wild type and mutant $\Delta bs3-3$ cultured for 5 days in Czapeck liquid medium. Arrows indicate the proteins identified by tandem mass spectrometry that differed in abundance by more than 1.5-fold between the wild type and mutant. **B**, Clustering analysis of the changes in protein secretion. Spots identified were clustered into two clusters (I and II) according to their percentage of volume, using the Pearson clustering algorithm. A bright red color indicates a high protein secretion value and a bright green color represents a low protein secretion value. **C**, Functional classification of downregulated proteins in cluster I.

cles were fusing with the plasma membrane (Fig. 6A). Further analysis showed that the extracellular activity of polygalacturonases and xylanases was significantly reduced in the mutant, but the transcription levels of the genes encoding these two hydrolases were not affected (Fig. 6D and E). These data suggest that the vesicle transport was blocked in the mutant. The results of Calcofluor white staining implied that the transport of polysaccharides, which were used for cell-wall synthesis, was impaired, resulting in the accumulation of polysaccharides at apical or subapical hyphal regions. This may partially explain the formation of swollen structures in mutants. The data indicate that *Bcsas1* can functionally regulate hyphal growth and sporulation by affecting extracellular secretion of proteins. *CLPT1*, a putative *SEC4* homologue in the filamentous fungus *Colletotrichum lindemuthianum*, was shown to be nonessential for hyphal elongation (Siriputthaiwan et al. 2005). Thus it appears that *SEC4* homologues may have different functions in different organisms.

We also found that *Bcsas1* disruption mutants exhibited different levels of virulence on different plant hosts. The pathogenicity of *Bcsas1* mutants was significantly impaired on tomato and apple fruit but completely lost on tomato leaves (Fig. 5). The reduced virulence in the *Bcsas1* mutants, compared with the wild type, may have been due to the lower growth rate (Fig. 3A and B) and reduced activity of cell wall-degrading enzymes as well as less protein secretion in general (Fig. 6). Polygalacturonase and xylanase have been shown to be required for virulence in *B. cinerea* (Brito et al. 2006; ten Have et al. 1998). In the current study, one-dimensional SDS-PAGE of extracellular protein extracts indicated that protein secretion was strongly impaired in the *Bcsas1* mutants (Fig. 6C). A comparative analysis of the secretome of the wild type and the *Bcsas1* deletion mutants provided further evidence that mutation of *Bcsas1* significantly depressed the secretion of polysaccharide hydrolases and proteases (Fig. 7). This finding is important for understanding the function of *Bcsas1* in regulating protein secretion in *B. cinerea*. Plant cell walls are mainly composed of polysaccharides such as pectin, cellulose, and hemicelluloses (Jamet et al. 2006). Among the nine polysaccharide hydrolases identified in our study, cellobiohydrolase (D1, BC1G_10880.1), α -galactosidase (D6, BC1G_00111.1), α -L-arabinofuranosidase (D8 and D9, BC1G_04994.1), and pectate lyase (BofuT4_P035890.1) have been reported to play an important role in cell-wall metabolism (English and Albersheim 1969; ten Have et al. 1998; Vu et al. 2012; Yajima et al. 2009). β -1,3-exoglucanase, a polysaccharide hydrolase downregulated in the secretome, has been reported to be involved in fungal morphogenesis by remodeling the β -1,3-glucan network of fungal cell wall or utilizing the extracellular β -1,3-glucan (Stubbs et al. 1999; Tamano et al. 2007). Although the secretion of one β -1,3-exoglucanase is obviously reduced in the mutants, the results of an agar plate assay indicated that the ability of the mutants to catabolize extracellular β -1,3-glucans was not significantly altered (data not shown). The function of this β -1,3-exoglucanase may be complemented by other members of this gene family.

Proteases are another group of enzymes that participate in the molecular dialogue between plants and pathogens (Espino et al. 2010). We found a greater level of secreted proteases and, in some cases, differential expression in the wild type compared with the *Bcsas1* mutant (Fig. 7). One of the identified proteases, a vacuolar protease A (D5, BC1G_06849.1), has been reported to be involved in spore morphogenesis and adaptation to nutritional stress in *S. cerevisiae* (Van den Hazel et al. 1996). In *Aspergillus fumigatus*, a vacuolar protease, ALP2, is involved in conidiogenesis (Reichard et al. 2000). Proteases have also been shown to play an important role dur-

ing the infection process. Fungal proteases could potentially contribute to plant cell-wall softening by degrading proteins, such as extensions, in the cell wall (Jamet et al. 2006). Proteases also contribute to the degradation of defense proteins secreted by host plants, thus facilitating infection. Furthermore, the amino acids released by the action of the proteases can be used to support fungal growth. Aspartic proteinase plays a crucial role in the pathogenesis of the fungal pathogen *Candida albicans* (Naglik et al. 2003). This protease was also identified as being differentially expressed in the *Bcsas1* mutant (Fig. 7). However, ten Have and associates (2010) reported that virulence was not affected in a mutant of *B. cinerea* containing a knockout of the aspartic proteinase gene *Bcap8*. Li and associates (2012) suggested that the loss of *Bcap8* activity in the knockout mutants may be partially compensated by expression of other *Bcap* family members.

In summary, the data suggest that *Bcsas1*, a *Rab8*- and *SEC4*-like gene in *B. cinerea*, plays an important role in hyphal growth and protein secretion by regulating vesicle transport. The mutation of *Bcsas1* greatly impaired protein secretion, thus resulting in lower levels of enzyme activity (hydrolases and proteases) in the extracellular medium. The reduction of growth rate and extracellular secretion resulted in decreased virulence of *B. cinerea*. Collectively, the data indicate that *Bcsas1* in *B. cinerea* is required for hyphal development and virulence. Future work will focus on the role of all ten *Rab* family genes in the regulation of virulence in *B. cinerea*.

MATERIALS AND METHODS

Fungal strains and culture conditions.

The *B. cinerea* haploid strain B05.10 was supplied by P. Tudzynski (Westfälische Wilhelms-Universität Münster, Germany) and was used as the wild-type strain. All strains were regularly incubated on PDA plates at 22°C. PDB and Czapeck liquid medium (per liter: 2.5 g of NaNO₃, 0.5 g of KCl, 0.5 g of MgSO₄·7H₂O, 10 mg of FeSO₄·7H₂O, 1 g of K₂HPO₄, 10 g of apple pectin) were used as liquid medium for collection of mycelia and extracellular proteins. SH agar medium containing 0.6 M sucrose, 5 mM HEPES, 1 mM (NH₄)₂HPO₄, and 1% agar was used to regenerate *B. cinerea* protoplasts. MEA plates (1% malt extract and 1.5% agar) were used for further selecting transformants.

Construction of the *Bcsas1* replacement vector and transformation.

For construction of the *Bcsas1* replacement vector, flank L, containing the 395-bp upstream sequence of the *Bcsas1* gene and the 428-bp *Bcsas1* gene sequence, was amplified by PCR with primer pair BSL-F and BSL-R from genomic DNA of *B. cinerea* B05.10; the *KpnI* and *BamHI* sites were added at the end of the primers, respectively (Supplementary Table S1). Flank R containing the 420-bp *Bcsas1* gene sequence and the 458-bp downstream sequence was amplified by PCR with primer pair BSR-F and BSR-R from genomic DNA; a *HindIII* site was added at both ends of this primer pairs. Plasmid pLOB7, provided by J. van Kan (Wageningen Agricultural University, The Netherlands), was used in the disruption of *Bcsas1*. It carries the hygromycin phosphotransferase gene *hph* under control of the *A. nidulans* *oliC* promoter and *trpC* terminator and confers resistance to hygromycin. Fragment L was digested by *KpnI* and *BamHI* and fragment R by *HindIII* and they were cloned into the corresponding site of the vector pLOB7, resulting in vector p Δ BS. Before transformation, the vector p Δ BS was linearized with *EcoRI*.

Conidia from sporulating cultures were collected and inoculated in PDB (10⁵ conidia ml⁻¹). Then the cultures were incu-

bated in a rotary shaker at 22°C and 160 rpm (HZQ-F100; HDL, Harbin, China) for 24 h. Mycelium was collected by filtration and was washed once with sterile water and twice with KC buffer (0.6 M KCl, 50 mM CaCl₂). The mycelium was suspended in 20 ml of 0.5% Glucanex (Sigma, St. Louis) solution (dissolved by KC buffer) and was incubated at 25°C and 100 rpm (HZQ-F100) for 2 h to generate protoplasts. The digested suspension was filtered by nylon membrane with 25 µm mesh size. Protoplasts were pelleted by centrifugation at 1,200 × g for 10 min, were washed twice, and were resuspended in KC buffer to a final concentration of 10⁸ protoplasts per milliliter. Protocols for transformation were slightly modified from an established procedure (Hamada et al. 1994). A suspension of 200 µl containing 2 × 10⁷ protoplasts was maintained on ice for 5 min, and subsequently, 100 µg of transformation vector dissolved in 100 µl of KC buffer (containing 2.5 mM spermidine) was added to the protoplasts and was incubated for 5 min on ice. Then 200 µl of 25% PEG 3350 (Sigma) in 50 mM CaCl₂, 10 mM Tris-HCl (pH 7.5) was added gently and the mixture was kept on ice for 20 min. After that, 400 µl of the same PEG solution was added, mixed gently, and incubated for 20 min at room temperature. Finally, the transformation mixture was transferred to 100 ml of SH agar (around 50°C), was mixed gently, and was poured into 10 petri dishes. After 20 h of incubation at 22°C, the regenerating protoplasts were overlaid with 5 ml of SH agar containing 50 µg of hygromycin B per milliliter. After incubating for 3 to 5 days at 22°C, the emerging colonies were transferred to MEA plates containing 70 µg of hygromycin B per milliliter for further selection. The transformants were verified via PCR with primer pair SJ-F and SJ-R. A 2.4-kb fragment was amplified by PCR from the transformants deriving from homologous integration. The positive transformants were further purified by single-spore isolation to obtain homokaryotic transformed strains. The purified transformants were analyzed by Southern blotting to exclude the transformants with ectopic integration sites.

DNA isolation and Southern blot analysis.

B. cinerea genomic DNA used for PCR was isolated according to the method described by Ceniz (1992). Genomic DNA used for Southern blot was extracted as described by Möller and associates (1992). The molecular probe used for DNA hybridization was amplified and labeled by PCR to incorporate digoxigenin-dUTP (Roche Applied Science, Indianapolis, IN, U.S.A.) into DNA fragment L with primers BSL-F and BSL-R. Hybridization was carried out in solution containing 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaPO₄, and 2 mM EDTA [pH 7.7]), 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.5% SDS, and 100 µg of denatured salmon sperm DNA per milliliter, at 65°C. Digoxigenin-labeled probe was detected by enzyme immunoassay and enzyme-catalyzed color reaction with NBT/BCIP (Roche Applied Science).

Detection of genes expression.

A suspension containing 10⁶ conidia of *B. cinerea* was inoculated in 20 ml of PDB and was incubated at 22°C and 160 rpm (HZQ-F100) for 8 h. H₂O₂ was then added into the medium to the final concentrations of 0, 5, and 10 mM. After incubating for 1 h, the germinating conidia were collected for RNA extraction. RNA was extracted from frozen germinating conidia ground in liquid nitrogen by Trizol reagent (Tiangen, Beijing). Samples containing 1 µg of RNA were treated with 1 U DNase I (Takara, Tokyo) for 30 min, and first-strand cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, U.S.A.).

Quantitative real-time PCR was carried out using Step One plus real-time PCR system (AB Applied Biosystems, Foster City, CA, U.S.A.) using SYBR green PCR master mix. Primer pairs were designed by Primer Express software 3.0 (AB Applied Biosystems). The PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 30 s. The change in fluorescence of SYBR green in every cycle was monitored by the system software, and the threshold cycle (C_t) over the background was calculated for each reaction. Samples were normalized using *Bcactin* and *Bctubulin* genes, and the relative expression levels were measured using the 2^(-ΔC_t) analysis method.

Infection assays.

The virulence of mutants was assayed on tomato fruits, apple fruits, and tomato leaves. Conidia of wild-type B05.10 and mutants were collected from 2-week-old cultures grown on PDA plates and suspended in a solution containing 10 mM KH₂PO₄ and 10 mM glucose at a final concentration of 5 × 10⁴ conidia per milliliter. Prewounded tomato and apple fruits were inoculated with a droplet (10 µl) of conidia suspension and were incubated in enclosed plastic trays to maintain a high relative humidity (95%) at 25°C. The detached tomato leaves (4-week-old) were slightly wounded with a hairbrush and were inoculated with 5 µl of conidia suspension. The leaves were then incubated in petri dishes at 25°C. Disease symptoms were scored every day.

Complementation of mutant.

With primer pair SC-F and SC-R, a 3,070-bp fragment was amplified by PCR, using Phusion high-fidelity DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.). This fragment, including the full length of the *Bcsas1* gene, 1,788 bp of the upstream sequence, and 292 bp of the downstream sequence. It was sequenced to ensure flawlessness. This fragment was then cloned into the *KpnI*-*BamHI* sites of pCA-sur vector (provided by Z. Ma, Zhejiang University, China), which contains a *sur* gene, conferring resistance to chlorimuron-ethyl, leading to a plasmid pΔBS-C to transform mutant strain. Transformation of mutants with the plasmid pΔBS-C was conducted as described above, except that chlorimuron-ethyl (50 µg ml⁻¹) was used as selection agent.

Fluorescence microscopy.

The tip of 3-day-old hypha was cut out from PDA plates and was carefully placed on microscope slides. Droplets (20 µl) of 5 µM FM4-64 (Invitrogen, Carlsbad, CA, U.S.A.) solution was dropped onto the hyphal tips and left for 10 s at room temperature. The hypha was then observed under fluorescence confocal microscope (LSM 510 META; Carl Zeiss, Oberkochen, Germany). The excitation was at 488 nm and emitted light was collected at 514 nm.

In order to compare cell wall differences, 3-day-old hyphae were stained with 100 µg Calcofluor white per milliliter and were observed under a Nikon Eclipse 800 epifluorescence microscope.

Preparation and relative abundance assay of extracellular proteins.

A suspension of 5 × 10⁶ conidia was inoculated into 100 ml Czapeck liquid medium. After culturing for 72 h, the filtrated supernatant was centrifuged at 20,000 × g at 4°C for 1 h to remove impurities, 0.03% (wt/vol) DOC (sodium deoxycholate) was then added to the supernatant, followed by careful mixing and incubation on ice for 30 min. Subsequently, the extracellular proteins were precipitated for 30 min on ice with ice-cold trichloric acetic acid at the final concentration of 10%

(wt/vol). The proteins were collected by centrifugation at $16,000 \times g$ at 4°C for 30 min and were washed three times with cold acetone. After air drying, the protein pellets were solubilized in 200 µl of lysis buffer (2 M thiourea, 7 M urea, 4% [wt/vol] CHAPS, 1% [wt/vol] dithiothreitol, and 2% [vol/vol] carrier ampholytes). For the relative abundance assay, the mycelium was separated from the supernatant and was dried to determine mass. The amount of supernatant used for protein extraction was adjusted, based on the dry mass obtained from the cultures. The protein samples were separated on 12% (wt/vol) SDS-PAGE gel and were silver stained.

Determination of extracellular polygalacturonase and xylanase activities.

Extracellular mediums cultured for 72 h were used for polygalacturonase and xylanase activity assays. For the polygalacturonase activity assay, pectin was used as substrate and the activity was expressed as the production of D-galacturonic acid per hour and per microgram of protein (Jiang et al. 2010). For the xylanase activity assay, xylan was used as substrate and the activity was expressed as the production of xylose per hour and per microgram of protein (Jeffries et al. 1998).

2-D gel electrophoresis and image analysis.

For 2-D gel electrophoresis, 250-µg aliquots of extracellular protein extracted from 5-day-old culture and resolved in 250 µl of lysis buffer were used to rehydrate gel strips (Immobiline Dry Strip, pH 4 to 7, 13 cm; GE Healthcare, Piscataway, NJ, U.S.A.) for 16 h. The 2-D electrophoresis and gel staining were carried out according to the method described by Li and associates (2010).

The stained gels were scanned using a flatbed scanner (Amersham Biosciences, Uppsala, Sweden) and were saved in TIF format. Image analysis was performed by Image Master 2-D Elite software (Amersham Biosciences). Three biological replicate gels resulting from three independent experiments were analyzed for *B. cinerea* wild type and $\Delta Bcsas1$ mutant. The amount of a protein spot was calculated based on the volume of that spot, and the spot volume was normalized as a percentage of the total volume of all spots. The normalized intensity of spots on three replicate 2-D gels was averaged, and a two-tailed nonpaired Student's *t*-test was used to determine whether the relative change was statistically significant between samples, using SPSS software (SPSS, Inc., Chicago). Protein spots whose secretion levels changed significantly were excised for protein identification.

In-gel digestion, MS, and database searching.

In-gel digestion was performed as described by Qin and associates (2011). Protein spots were excised from the 2-D gels and were destained with 50 mM NH_4HCO_3 in 50% (vol/vol) methanol for 1 h at 40°C. After completely drying in a vacuum centrifuge, the gel pieces were digested at 37°C for 16 h with 10 ng of trypsin per microliter. Digested peptides were extracted by three changes of 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile, were lyophilized, and were submitted to matrix-assisted laser desorption-ionization time of flight (MALDI-TOF)/TOF MS/MS.

For MALDI-TOF/TOF MS/MS analysis, peptides were resuspended with 10 µl of 70% acetonitrile containing 0.1% TFA, and 1 µl was taken and spotted onto the AnchorChip MALDI target plate (Bruker Daltonics, Billerica, MA, U.S.A.). Then, 1 µl of matrix solution (1 mg ml^{-1} , α -cyano-4-hydroxycinnamic acid in 70% acetonitrile containing 0.1% TFA) was spotted after the sample solution dried. Mass spectra were acquired on a MALDI-TOF/TOF mass spectrometer (UltrafleXtreme; Bruker Daltonics). The instrument was operated in the positive

reflection mode and was externally calibrated using the peptide calibration kit (Bruker Daltonics). MS spectra were acquired with 400 laser shots per spectrum, whereas MS/MS spectra were obtained using 1,500 laser shots per fragmentation spectrum. To acquire the MS/MS fragmentation spectra, the 15 strongest peaks of each MS spectra were selected as precursor ions, which excluded trypsin autolytic peptides and other known background ions.

For database searching, MS data were uploaded with Bio-tools software (ver. 3.2; Bruker Daltonics) to Mascot for database searching on the Matrix Science public web site and were searched against NCBI nr protein databases (version 20120107; 16,831,682 sequences and 5,781,564,572 residues). Search parameters were set as fungi; proteolytic enzyme, trypsin; max missed cleavages, 1; fix modifications, carbamidomethyl (C); variable modifications, oxidation (M); peptide mass tolerance, 100 ppm; fragment mass tolerance, 0.5 Da. Only significant hits as defined by Mascot probability analysis were considered.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Broad Institute *Botrytis cinerea* genome database:
www.broad.mit.edu/annotation/genome/botrytis_cinerea
 Matrix Science web site: www.matrixscience.com
 Pathogen-Host Interactions database: www.phibase.org