Actin Is Required for Cellular Development and Virulence of Botrytis cinerea via the Mediation of Secretory Proteins

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ABSTRACT  Actin is a vital component of the cytoskeleton of living cells and is involved in several complex processes. However, its functions in plant-pathogenic fungi are largely unknown. In this paper, we found that deletion of the Botrytis cinerea actin gene bcactA reduced growth and sporulation of B. cinerea and lowered virulence. Based on iTRAQ (isobaric tags for relative and absolute quantification)-based proteomic analysis, we compared changes of the secretome in ΔbcactA and wild-type strains. A total of 40 proteins exhibited significant differences in abundance in ΔbcactA mutants compared with the wild type. These proteins included 11 down-accumulated cell wall-degrading enzymes (CWDEs). Among them, two CWDEs, cellobiohydrolase (BcCBH) and β-endoglucanase (BcEG), were found to contribute to the virulence of B. cinerea, indicating that bcactA plays a crucial role in regulating the secretion of extracellular virulence factors. These findings unveil previously unknown functions of BcactA to mediate the virulence of B. cinerea and provide new mechanistic insights into the role of BcactA in the complex pathogenesis of B. cinerea.

IMPORTANCE  The cytoskeleton is an important network that exists in cells of all domains of life. In eukaryotic cells, actin is a vital component of the cytoskeleton. Here, we report that BcactA, an actin protein in B. cinerea, can affect the growth, sporulation, and virulence of B. cinerea. Furthermore, iTRAQ-based proteomic analysis showed that BcactA affects the abundance of 40 extracellular proteins, including 11 down-accumulated CWDEs. Among them, two CWDEs, cellobiohydrolase (BcCBH) and β-endoglucanase (BcEG), contributed to the virulence of B. cinerea, indicating that bcactA plays a crucial role in regulating extracellular virulence factors. These findings unveil previously unknown functions of BcactA in mediating growth, sporulation, and virulence of B. cinerea.

KEYWORDS  actin, Botrytis cinerea, pathogenesis, secretome

The cytoskeleton is an extremely highly organized but complex and dynamic network that exists in cells of all domains of life, including archaea, bacteria, and eukaryotes. In eukaryotic cells, it is mainly composed of microfilaments, microtubules, and intermediate filaments. Actin is a vital component of microfilaments, and many actin isoforms exist. In mammals, there are three types of actin, namely, α-actin, β-actin, and γ-actin. Deficient mutants of some actin isoforms are lethal, while others are viable. In mice, whole-body β-actin-knockout (Actb−/−) mutants are lethal, while whole-body γ-actin-null (Actg1−/−) mutants are not (1). In plants, several actin proteins have been discovered. Misexpression of act1 in vegetative tissues can cause dwarfing of Arabidopsis thaliana (2), while the act2−1 mutant exhibits no phenotypic distinction from the

Citation  Li H, Zhang Z, Qin G, He C, Li B, Tian S. 2020. Actin is required for cellular development and virulence of Botrytis cinerea via the mediation of secretory proteins. mSystems 5:e00732-19. https://doi.org/10.1128/mSystems.00732-19.

Editor  Jack A. Gilbert, University of California San Diego

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Received 31 October 2019
Accepted 31 January 2020
Published 25 February 2020
wild type (WT) when grown in soil (3). In filamentous fungi, three high-order F-actin structures exist with distinct functions: actin patches, cables, and rings. Actin patches are peripheral punctate structures present at subapical regions, where the endocytic machinery is located (4). The localization of actin patches is indicative of actin’s function in endocytosis and exocytosis at the hyphal tip and in coupling those functions to maintain tip growth (5–7). Actin cables predominantly localize at the apex of hyphae and form tracks for myosin V-dependent polarized secretion and organelle transport (8–10). Actin rings participate in septum formation and are essential for cytokinesis in budding yeast and pathogenesis in Magnaporthe oryzae (11–13). Actin or actin-related proteins are widely involved in the pathogenicity and secretion of fungi. The F-actin capping protein is important for the hyphal growth and virulence of Botrytis cinerea (14). Disruptions of the actin cytoskeleton in Aspergillus nidulans can lead to the inhibition of enzyme secretion via blockade of secretory vesicle transportation (15).

B. cinerea is a major phytopathogenic fungus that has been classified as the second most devastating plant pathogen (16). It invades more than 1,000 plant species, particularly fresh horticultural crops, during cultivation, storage, and distribution, leading to huge economic losses of $10 to $100 billion annually worldwide (16–18). In the past few years, significant efforts have been focused on effective control by exploring the molecular mechanisms of the pathogenicity of B. cinerea. The secretory proteins of B. cinerea comprise various virulence factors that facilitate successful host tissue penetration and colonization (19). Our previous studies have shown that B. cinerea is able to secrete a variety of enzymes and metabolites that can kill host cells during the infection process (20) and even adjust its secretome in response to changes in ambient pH values (21). The Rab family protein Bcsas1 also plays an important role in hyphal growth and virulence regulation in B. cinerea by affecting vesicle transport and protein secretion (22). Therefore, secretome analysis can provide new insights into the role of extracellular proteins in pathogenic invasion. Considering that actin is a cytoskeleton protein reportedly involved in the secretion of enzymes secretion of A. nidulans (15), it may also be involved in the secretion of virulence factors in B. cinerea. Among the actin proteins, the expression of BcactA was observed to be increased for the first 4 days when bean leaves were inoculated with B. cinerea and thereafter decreased (23).

Proteomics have played a dominant role in the study of specific proteins involved in the virulence of plant pathogens (24). Although next-generation and third-generation high-throughput sequencing have developed rapidly and have a strong advantage in determining global changes at the transcription level, they are insufficient in predicting changes at the protein level, because divergence exists between gene transcription and protein expression. Besides gene transcription, protein expression can also be affected by transcription regulation and protein translation (25). In addition, proteomics, such as isobaric tags for relative and absolute quantification (iTRAQ)-based quantitative proteomic analysis, is more efficient in investigating the subproteome, as proteins can be carefully isolated before analysis. We have successfully used proteomics in the past to investigate the subproteome of both nuclear proteins in tomato fruits at different ripening stages (26) and tonoplast proteins in apple fruits at different periods of senescence (27).

In this study, the mutant of the cytoskeleton protein BcactA was constructed. Deletion of bactA resulted in reduced growth rate, sporulation, and virulence of B. cinerea. To further unveil the downstream proteins regulated by BcactA, iTRAQ-based quantitative proteomic analysis was conducted. Forty differentially expressed secretory proteins were identified as being associated with the ΔbactA mutant. Among them, BcCBSH and BcEG were shown to be involved in the regulation of B. cinerea virulence. These results reveal a previously unknown function of actin to control both the virulence of a phytopathogen and its associated involved mechanisms.

RESULTS

BcactA is required for vegetative growth and sporulation. The BcactA gene (Bcin16g02020) encodes a conserved actin protein. The amino acid sequence of BcactA
shows high homology with the actin proteins in model organisms (see Fig. S1 in the supplemental material). To investigate the role of BcactA in the growth, colony morphology, and sporulation of \textit{B. cinerea}, a knockout mutant of \textit{bcactA} was generated by replacing the \textit{bcactA} gene with the hygromycin resistance cassette through transformation of the protoplasts of the wild-type (WT) strain. (B) PCR diagnosis of \textit{ΔbcactA} mutants. PCR was performed using the primer pair \textit{bcactA-homo-up}/HPH-det, to ensure that homologous recombination occurred at the target site. (C) Diagnosis of the homozygotes of \textit{ΔbcactA} mutants. PCR was performed with the primer pair \textit{bcactA-homo-up}/bcactA-R-down, to ensure that the mutants were homozygous with no wild-type bands. (D) Southern blot analysis of WT and \textit{ΔbcactA} strains. The genomic DNA was digested with SacI and BamHI, separated in an agarose gel, and hybridized with a probe (a fragment on the hygromycin resistance cassette labeled with digoxigenin). Numbers represent different strains. (E) Immunoblotting analysis of BcactA protein in WT and \textit{ΔbcactA} strains. Total cytoplasmic proteins were extracted from 2-day-old mycelia cultured in PDB and immunoblotted with commercially available antibody to actin (Abmart; M20011). Histone 3 (H3) was used as a loading control.

\textbf{FIG 1} Generation of \textit{ΔbcactA} mutants. (A) Replacement strategy for deletion of the \textit{bcactA} gene. The 5’ flank and 3’ flank of \textit{bcactA} were amplified and ligated into pLOB7 to flank the hygromycin resistance cassette to obtain the replacement vectors. Deletion mutants were generated by replacing the \textit{bcactA} gene with the hygromycin resistance cassette through transformation of the protoplasts of the wild-type (WT) strain. (B) PCR diagnosis of \textit{ΔbcactA} mutants. PCR was performed using the primer pair \textit{bcactA-homo-up}/HPH-det, to ensure that homologous recombination occurred at the target site. (C) Diagnosis of the homozygotes of \textit{ΔbcactA} mutants. PCR was performed with the primer pair \textit{bcactA-homo-up}/bcactA-R-down, to ensure that the mutants were homozygous with no wild-type bands. (D) Southern blot analysis of WT and \textit{ΔbcactA} strains. The genomic DNA was digested with SacI and BamHI, separated in an agarose gel, and hybridized with a probe (a fragment on the hygromycin resistance cassette labeled with digoxigenin). Numbers represent different strains. (E) Immunoblotting analysis of BcactA protein in WT and \textit{ΔbcactA} strains. Total cytoplasmic proteins were extracted from 2-day-old mycelia cultured in PDB and immunoblotted with commercially available antibody to actin (Abmart; M20011). Histone 3 (H3) was used as a loading control.

\textbf{Role of Actin in \textit{B. cinerea}}. To investigate the role of BcactA in the growth, colony morphology, and sporulation of \textit{B. cinerea}, a knockout mutant of \textit{bcactA} was generated by replacing the \textit{bcactA} gene (Bcin16g02020) with a hygromycin resistance cassette through transformation of protoplasts of the wild-type strain (Fig. 1A). Three independent transformants were obtained through screening on selection medium supplemented with hygromycin B and subsequent PCR verification (Fig. 1B). After single spore isolation, the transformants were verified by PCR as homozygous (Fig. 1C) and further confirmed to be single-copy insertions by Southern blot analysis (Fig. 1D). To further confirm that the mutant indeed lost the actin protein, we conducted Western blot analysis with a commercial antibody to actin. The results indicated that in the \textit{ΔbcactA} knockout mutant, the actin protein was completely lost (Fig. 1E and Fig. S2). To confirm the correlation between the phenotype of the \textit{ΔbcactA} mutant and the inactivation of \textit{bcactA}, complemented strains were constructed and verified by PCR (Fig. S3). The growth rate of the \textit{ΔbcactA} mutant was reduced compared with that of the wild-type and complemented strains on complete medium (CM). The colony diameter of the mutant exhibited a 34\% reduction at 24 h postinoculation (hpi), and a 36\% reduction at 48 hpi, relative to the wild-type and complemented strains (Fig. 2B). The hyphae of the \textit{ΔbcactA} mutant were more compact than those of the wild-type and complemented strains on CM (Fig. 2A). The sporulation of the \textit{ΔbcactA} mutant was significantly reduced. After culture for 10 days on CM, the sporulation of the mutant was only 33\% of that of the wild-type and complemented strains (Fig. 2D). The mutant spores were more likely to be distributed around the center of the plates, while the spores of the wild-type and complemented strains were more evenly distributed over the entire plates (Fig. 2C). These results show that BcactA plays a role in the growth, colony morphology, and sporulation of \textit{B. cinerea}.

\textbf{BcactA participates in regulating the pathogenicity of \textit{B. cinerea}}. To determine whether BcactA was involved in the regulation of pathogenicity in \textit{B. cinerea}, apple and
tomato fruits, as well as detached tomato leaves, were inoculated with the spores of ΔbcactA mutants. ΔbcactA mutants exhibited reduced virulence in different hosts (Fig. 3). At 96 hpi, apples inoculated with the ΔbcactA1 mutant showed no lesions, while the WT-inoculated apples showed an average lesion size that reached 11 mm (Fig. 3A). Similarly, considerably reduced lesion sizes were observed in the ΔbcactA1 mutant-inoculated tomato fruits in comparison with the WT-inoculated tomato fruits (Fig. 3B). At 48 hpi, lesion size was approximately 4 mm in the ΔbcactA1 mutant-inoculated tomato leaves, compared with 8 mm in the WT-inoculated tomato leaves (Fig. 3C). The complemented strain of the ΔbcactA1 mutant exhibited almost the same level of virulence as the WT (Fig. 3D). These results show that BcactA plays a role in the pathogenesis of B. cinerea.

BcactA affects the contents of extracellular proteins. To investigate the downstream targets of BcactA, extracellular proteins were extracted and iTRAQ-based quantitative proteomic analysis was used to analyze the secretome of the WT and ΔbcactA strains. Proteins were labeled with iTRAQ isobaric tags and subjected to nano-liquid chromatography–tandem mass spectrometry (nanoLC-MS/MS) analysis (Fig. 4). Totals of 220 and 232 proteins with a global false-discovery rate (FDR) below 1% were identified in the two biological replications, respectively, when searching against the B. cinerea protein database. A 2-fold cutoff was used as a determinant for proteins showing a significant change in abundance. The abundances of 58 and 63 proteins showed significant changes in the ΔbcactA mutant in the two biological replications, respectively, in comparison with the WT (40 proteins in common) (Fig. 5A). The up-accumulated and down-accumulated proteins in ΔbcactA mutants were classified into different functional categories, according to the blast2go tool, which included cell wall-degrading enzymes (CWDEs), oxidation-reduction proteins, glycosylation proteins, carbohydrate metabolism, resistance-associated proteins, and other functional proteins. The CWDEs were the most highly represented group among the categories (Fig. 5B). The ratios of iTRAQ reporter ion intensities, along with all relevant identification information for the differentially expressed proteins, are listed in Table 1.
CWDEs were decreased in ΔbcactA mutants. To further analyze the 40 differentially expressed proteins identified in the ΔbcactA mutants, a heat map within each functional category was applied according to the expression patterns of identified proteins (Fig. 5C). Noticeably, 11 CWDEs were down-accumulated, except pectate lyase (BcPL; Bcin15g00520). The 11 CWDEs included mannosidase (BcMAN; Bcin01g05680), α-amylase (BcAMY; Bcin04g06250), glycoside hydrolase (BcGH; Bcin7g02860), β-1,3-exoglucanase (BcEXG; Bcin11g01370), glucoamylase (BcGA; Bcin04g04190), arabinofuranosidase (BcABF; Bcin13g03950), cellobiohydrolase (Bc-
CBH; Bcin13g02100), endoglucanase (BcEG; Bcin09g00200), β-1,4-glucan cellobiohydrolase b (BcCBH-b; Bcin16g03950), α-L-rhamnosidase (BcRHA; Bcin01g11040), and esterase (BcEST; Bcin15g00520) (Fig. 5C). These results indicate that BcactA affected the level of CWDEs.

**Functional analysis of the identified CWDEs.** Among the 11 down-accumulated CWDEs, BcEST reportedly has no effect on the penetration or pathogenicity of *B. cinerea* (28). To further investigate the functions of the other 10 CWDEs (BcMAN, BcAMY, BcGH, BcEXG, BcGA, BcABF, BcCBH, BcEG, BcCBH-b, and BcRHA), knockout mutants of each gene were generated and verified to be homozygous mutants (Fig. S4 and S5). The growth rates of Δbcman, Δbcamy, Δbcgh, Δbcexg, Δbcga, Δbcabf, Δbceg, Δbcbhb- and Δbcrha mutants were almost the same as that of the WT, and only the growth of the Δbcbhb mutant was slower than that of the WT (Fig. 6A). Sporulation of the Δbcbhb mutant exhibited a significant reduction; however, that of other mutants showed no significant difference from the WT (Fig. 6B). Similarly, no significant differences were noted in colony morphology of all mutants in comparison with the WT, indicating that BcCBH affected hyphal growth of *B. cinerea.*

**BcCBH and BcEG are involved in the regulation of virulence in *B. cinerea***. To evaluate whether the 10 CWDEs were involved in the regulation of virulence of *B. cinerea*, spores of the mutants were collected and inoculated into apple fruits and detached tomato leaves. Compared with the WT-inoculated tomato leaves, lesion diameters were significantly smaller in the Δbcbhb mutant-inoculated tomato leaves (Fig. 7B). However, no significant differences were noted in lesion diameters on the Δbcbhb mutant-inoculated apple fruits (Fig. 7A). Nevertheless, the Δbceg mutant exhibited a significant reduction in lesion diameters in the apple fruits (Fig. 7D) but not in detached tomato leaves (Fig. 7E). To confirm that virulence reduction of the mutants was due to the inactivation of corresponding genes, complemented strains were constructed and verified by PCR (Fig. S3). The complemented strains of the Δbcbhb and Δbceg mutants were able to infect tomato leaves and apple fruits at the same rate as the WT (Fig. 7C and F). No significant differences in virulence were observed in other mutants in comparison with the WT (Fig. S6 and S7). These results suggested that two CWDEs, BcCBH and BcEG, were important for the pathogenesis of *B. cinerea.*
DISCUSSION

The cytoskeleton of living cells is a highly organized and dynamic network, which plays important roles in various cellular functions, including the regulation of cell polarity, endocytosis, protein secretion, septation, and organelle transport. In fungi, there are three forms of cytoskeleton, filamentous actin, microtubules, and septins, among which actin is a vital component. Actin exists in two forms, G-actin monomers and functional filamentous actin (F-actin). The transition to either of the two forms is closely related to its function (29). An NADPH-oxidase ortholog in yeast, Yno1p, was demonstrated to regulate actin cable formation in yeast cells (30). The regulatory subunit of NADPH-oxidase (NoxR) is required for the formation of a toroidal F-actin structure, which is essential for the protrusion of a rigid penetration peg in M. oryzae (11). In our previous study, we found that the protein abundance of actin was affected by NoxR in B. cinerea (31). Moreover, gene expression levels of eight NADPH oxidase (NOX) subunits, including bcnoxR, were higher in the ΔbcactA mutant (see Fig. S8 in the supplemental material), indicating that a complex and close association exists between BcnoxR and BcactA. The ΔbcactA mutant exhibited a reduction in growth rate, sporulation, and virulence in apple and tomato fruits and in detached tomato leaves (Fig. 2 and 3). The visibility of the ΔbcactA mutant was perhaps due to the functional redundancy of other actin-related proteins or other cytoskeletal forms.

FIG 5 Analysis of differentially accumulated extracellular proteins in ΔbcactA mutants. (A) Venn diagram of differentially expressed proteins. ΔbcactA1 refers to the results of the first biological replication, while ΔbcactA2 refers to the results of the second biological replication. (B) Functional categories of up-accumulated and down-accumulated proteins in ΔbcactA mutant. (C) Expression patterns of proteins within each functional category. The logarithm of the protein ratio was used to measure the changes in protein abundance. Each row in the color heat map indicates a single protein. The gene identifiers (Bcin numbers) and functional annotations are shown. Red and green colors indicate up-accumulated and down-accumulated proteins, respectively, in ΔbcactA mutants versus WT.
| Function and accession no. | Protein description | Log (ΔbcactA<sup>1</sup>:WT)<sup>a</sup> | Log (ΔbcactA<sup>2</sup>:WT)<sup>b</sup> | Unused protein score | Peptide (95%)<sup>c</sup> | % sequence coverage<sup>d</sup> |
|---------------------------|---------------------|-----------------|-----------------|-------------------|------------------|-----------------|
| **Cell wall degradation** |                     |                 |                 |                   |                  |                 |
| Bcin09g00200              | β-Endoglucanase     | -0.40           | -0.57           | 21.40             | 24               | 37.53           |
| Bcin16g03950              | β-1,3-Glucan celllobiohydrolase protein | -0.62           | -0.68           | 13.63             | 9                | 32.44           |
| Bcin04g06250              | α-Amylase           | -0.95           | -1.04           | 48.98             | 47               | 53.20           |
| Bcin13g02100              | Celllobiohydrolase  | -0.32           | -0.38           | 84.18             | 81               | 68.41           |
| Bcin07g02860              | Glycose hidrolase   | -0.70           | -0.90           | 8.02              | 5                | 15.06           |
| Bcin01g05680              | Mannosidase         | -0.43           | -0.30           | 17.91             | 9                | 16.98           |
| Bcin01g11040              | α-L-Rhamnosidase    | -0.38           | -0.54           | 33.32             | 24               | 32.35           |
| Bcin13g09350              | Arabinosidase       | -0.62           | -0.40           | 92.26             | 95               | 73.12           |
| Bcin11g01370              | β-1,3-Exoglucanase  | -0.58           | -0.61           | 204.56            | 215              | 72.17           |
| Bcin15g00520              | Esterase            | -1.26           | -1.15           | 17.94             | 20               | 48.61           |
| Bcin04g04190              | Glucoamylase        | -0.59           | -0.57           | 83.15             | 68               | 59.84           |
| Bcin03g05820              | Pectate lyase       | 1.32            | 1.32            | 16.94             | 16               | 50.76           |
| **Oxidation-reduction**  |                     |                 |                 |                   |                  |                 |
| Bcin16g02560              | Glutaminase         | -0.47           | -0.51           | 57.49             | 52               | 57.68           |
| Bcin03g01920              | Peroximal catalase  | 0.78            | 0.78            | 33.78             | 26               | 43.03           |
| Bcin03g01540              | GMC oxidoreductase  | 1.56            | 1.65            | 354.50            | 418              | 84.87           |
| Bcin16g04640              | Formate dehydrogenase | 0.74         | 1.28            | 41.14             | 27               | 51.72           |
| Bcin02g07080              | Choline dehydrogenase | 1.14          | 1.29            | 131.40            | 127              | 57.82           |
| Bcin06g01180              | Catalase isozyme P protein | 0.66     | 1.09            | 80.40             | 66               | 60.63           |
| **Glycosylation**         |                     |                 |                 |                   |                  |                 |
| Bcin01g07210              | α-Mannosidase family protein | -0.71 | -0.56         | 15.71             | 10               | 17.53           |
| Bcin01g03390              | α-Mannosidase protein | 0.68      | 0.64            | 23.99             | 16               | 16.96           |
| **Carbohydrate metabolism** |                     |                 |                 |                   |                  |                 |
| Bcin12g03690              | 3-Carboxymuconate cyclase | -0.59       | -0.50           | 0.97              | 1                | 4.14            |
| Bcin02g02750              | Citrate synthase    | 1.03            | 1.29            | 17.09             | 11               | 30.08           |
| Bcin02g05920              | Aconitate hydratase | 0.88            | 0.96            | 20.51             | 14               | 19.54           |
| Bcin07g02610              | 6-Phosphogluconate dehydrogenase | 0.54     | 1.17            | 16.69             | 15               | 25.48           |
| Bcin02g06400              | Dihydrolipoyl dehydrogenase | 0.38       | 0.79            | 16.71             | 9                | 22.16           |
| Bcin01g06450              | Enolase             | 0.77            | 1.17            | 59.08             | 42               | 63.47           |
| Bcin14g01980              | Transketolase       | 0.90            | 1.30            | 39.63             | 22               | 32.41           |
| Bcin09g01320              | Isocitrate lyase    | 0.54            | 0.96            | 18.01             | 11               | 24.09           |
| Bcin15g04970              | Glucose-6-phosphate isomerase | 0.53   | 0.96            | 29.94             | 28               | 35.70           |
| **Resistance associated** |                     |                 |                 |                   |                  |                 |
| Bcin02g04640              | Avr-Pi54 protein    | 1.45            | 1.72            | 153.40            | 171              | 84.13           |
| Bcin01g10500              | Cyanovirin-N family protein | 0.82      | 0.82            | 15.09             | 14               | 81.13           |
| **Other functions**       |                     |                 |                 |                   |                  |                 |
| Bcin14g00390              | Hypothetical protein | -1.51       | -1.17           | 29.71             | 25               | 34.75           |
| Bcin01g09390              | Serine-rich protein | -0.36           | -0.72           | 19.73             | 15               | 34.05           |
| Bcin03g05720              | Hypothetical protein | -0.86       | -1.14           | 16.00             | 17               | 58.71           |
| Bcin08g02990              | Hypothetical protein | -0.83       | -0.54           | 38.29             | 34               | 46.26           |
| Bcin05g03460              | Hypothetical protein | -0.51       | -0.72           | 7.72              | 7                | 21.16           |
| Bcin02g01060              | Nucleoside diphosphate kinase protein | 0.92   | 0.88            | 10.10             | 6                | 31.22           |
| Bcin03g01490              | Dihydrolipicolinate synthase protein | 0.47  | 0.82            | 2.38              | 2                | 7.08            |
| Bcin04g06570              | Cobalamin-independent methionine synthase | 0.61  | 1.26            | 21.30             | 15               | 20.60           |
| Bcin01g05720              | Serine-rich protein | 1.16            | 1.16            | 102.30            | 100              | 57.48           |

<sup>a</sup>Accession number from Blast search on NCBI.

<sup>b</sup>The logarithm of the fold change of protein expression levels in ΔbcactA mutant versus the wild type from the first independent biological replicate. The meaningful cutoff was fixed at 2-fold corresponding to the logarithm of the iTRAQ ratio of >0.3 for upregulation and <−0.3 for downregulation, as mentioned in Materials and Methods.

<sup>c</sup>The logarithm of the fold change of protein expression levels in ΔbcactA mutant versus the wild type from the second independent biological replicate. The meaningful cutoff was fixed at 2-fold corresponding to the logarithm of the iTRAQ ratio of >0.3 for upregulation and <−0.3 for downregulation, as mentioned in Materials and Methods.

<sup>d</sup>Number of unique peptides identified (95% confidence).

<sup>e</sup>Amino acid sequence coverage for the identified proteins.
Actin plays a role in cell motility, maintenance of cell shape, and secretion (32, 33). F-actin cables extend from the plasma membrane into the cell and provide tracks for the targeted delivery of secretory vesicles (32, 33). In pathogenic fungi, the secretory proteins comprise virulence factors that facilitate successful host tissue penetration and colonization (19). Secretory proteins are important for the virulence of B. cinerea, as its appressorium is not rigid enough to rupture the host cell wall (34). B. cinerea utilizes different secretory proteins to invade host cells, depending on environmental conditions, such as differences in pH values. At pH 4, the secretome of B. cinerea favors proteolysis, which is essential for the degradation of the antifungal proteins secreted by the plant host (21, 35). At pH 6, the secretome of B. cinerea includes more CWDEs, which are helpful in decomposing the host cell wall to achieve full virulence (21, 36). Therefore, secretome analysis can provide new insights into extracellular protein

**FIG 6** Phenotypic analysis of deletion mutants of 10 cell wall-degrading enzymes (CWDEs). (A) Colony morphology and colony diameters of wild-type (WT) and 10 CWDE mutant strains after 48 h of growth on complete medium (CM) plates. (B) Sporulating cultures and statistical analysis of sporulation of WT and 10 CWDE mutant strains after 10 days of growth on CM plates. Strains were cultured at 22°C. Data presented are the mean ± SD (n = 3). An asterisk indicates a significant difference from the WT (P < 0.05). bcman, mannosidase; bcamy, α-amylase; bcgh, glycoside hydrolase; bcexg, β-1,3-exogluca-nase; bcga, glucoamylase; bcabf, arabinofuranosidase; bcbbh, cellobiohydrolase; bceg, endoglucanase; bcbbh-b, β-α-glucan cellobiohydrolase b; bcra, α-L-rhamnosidase.
function in pathogenic invasion. The iTRAQ-based quantitative proteomic analysis revealed characteristics such as high resolution, high throughput, accurate protein quantification, and repeatability (37). The technique has been used to characterize the dynamic changes of tonoplast proteins during apple fruit senescence (27). In this study, the secretome of the WT and ΔbcactA strains was examined through iTRAQ-based quantitative proteomic analysis, and 40 differentially expressed proteins were identified in the ΔbcactA mutant (Fig. 5). These proteins included oxidation-reduction-related proteins, resistance-associated proteins, and cell wall-degrading proteins, among others. Oxidation-reduction proteins are considered to be associated with pathogen invasion, because an oxidative burst usually occurs during infection (34, 38). We identified several oxidation-reduction proteins, including a Cu-Zn superoxide dismutase (Bcin03g03390) that was for the first time determined by proteomics. A deletion mutant of this gene results in reduced virulence in the host (39).

The CWDEs are important in the pathogenesis of phytopathogenic fungi (24). The plant cell wall, which is mainly composed of cellulose, xylan, pectin, and other polysaccharides, is an important barrier for effective defense against phytopathogenic fungi. The composition of the cell wall differs significantly among plant lineages and plant tissues (36). To penetrate the plant cell wall, phytopathogenic fungi produce a diverse array of CWDEs that are capable of degrading the main structural polysaccharide components of the cell wall (40). A total of 1,155 predicted genes encode the enzymes responsible for the creation, modification, or degradation of glycosidic bonds in B. cinerea, and 275 of those are predicted to be secretory proteins, as they contain extracellular signal peptide sequences (18). These secretory proteins have been fre-
quently detected in comparative proteomics and are supposed to be essential virulence proteins (21, 41, 42). A variety of CWDEs have been proven to be essential for the virulence of B. cinerea. Endopolygalacturonase 1 (BcPG1) is involved in lesion expansion on apple fruits, tomato fruits, and leaves, while endopolygalacturonase 2 (BcPG2) is involved in both primary infection and lesion expansion of B. cinerea on tomato fruits and broad beans (43, 44). In addition, endo-β-1,4-xylanase (BcXYN11A), which can degrade xylan in the plant cell wall, is involved in the virulence of B. cinerea (45). In this study, we identified 11 CWDEs that were down-accumulated in the extracellular proteins of the ΔbcactA mutant (Fig. 5C). Cutinase A (BccutA) was previously reported as not essential for penetration of gerbera and tomato (28). The other 10 CWDEs are glycoside hydrolases (GHs), which are mainly associated in cellulose and hemicellulose degradation. We investigated the functions of the 10 GHs encoded by genes associated with growth, morphogenesis, and virulence of B. cinerea. The results indicated that two proteins, BcCBH and BcEG, were involved in the virulence of B. cinerea. The Δbcchb mutant showed a reduction in growth rate, sporulation, and virulence on detached tomato leaves, while the Δbceg mutant exhibited reduced virulence in apple fruits (Fig. 6 and 7). Other mutants showed almost the same virulence as the wild type, perhaps due to the redundancy of function provided by other enzymes, considering the large number of CWDEs in B. cinerea.

In conclusion, we found that the actin protein BcactA mediates growth, development, and virulence of B. cinerea, as well as the associated mechanisms. The secretion of a large number of extracellular CWDEs, including some critical virulence factors, is regulated by BcactA, which eventually affects the pathogenicity of B. cinerea (Fig. 8). These findings are beneficial for our understanding of the role of BcactA in the complex pathogenesis of B. cinerea and provide a novel insight into the regulation network of the actin cytoskeleton.

**FIG 8** The mode of action of actin (BcactA) regulating the growth, development, and virulence of B. cinerea. BcnoxR regulates the actin cytoskeleton, and BcactA affects the secretion of extracellular proteins, which are divided into six groups according to their functions. Among 12 cell wall-degrading enzymes; OR, oxidation-reduction proteins; G, glycosylation proteins; CM, carbohydrate metabolism; R, resistance proteins; OF, other function proteins; BcMAN, mannosidase; BcAMY, α-amylase; BcGH, glycosidase hydrolase; BcEXG, β-1,3-exoglucanase; BcGA, glucoamylase; BcABF, arabinofuranosidase; BcCBH, cellobiohydrolase; BcEG, endoglucanase; BcCBH-b, β-β-glucan cellobiohydrolase b; BcRHA, α-L-rhamnosidase; ROS, reactive oxygen species.
**MATERIALS AND METHODS**

**Fungal strains and culture conditions.** *B. cinerea* (B05.10) was used as the recipient strain for the transformation experiments and as the wild-type control. The wild type and deletion mutants of *B. cinerea* were normally maintained at 22°C on CM, which contained 10 g glucose, 2 g peptone, 1 g yeast extract, 1 g Casamino Acids, 6 g NaNO₃, 0.5 g KCl, 1.11 g MgSO₄·7H₂O, 1.5 g KH₂PO₄, and 0.05 g yeast nitrogen base in 1 liter of distilled water. For collection of spores, the strains of *B. cinerea* were first cultured on CM plates for 1 week at 22°C. Spores were then collected and diluted with sterile distilled water to 1 × 10⁷ spores ml⁻¹, by counting with a hemocytometer under a microscope. For extracellular protein isolation, the spore suspension (1 ml) was added to potato dextrose broth (PDB; which contained 200 g peeled potatoes and 20 g glucose in 1 liter of distilled water) medium (100 ml) and cultured at 22°C for 24 h with shaking at 180 rpm. The mycelium was then harvested using four layers of gauze, washed thoroughly with sterile distilled water, and transferred to modified Czapek medium which contained 1% pectin from apples (Sigma, St. Louis, MO, USA), 0.2% NaNO₃, 0.1% K₂HPO₄, 0.05% KCl, 0.05% MgSO₄·7H₂O, and 0.036 mM FeSO₄·7H₂O. After incubation at 22°C for 72 h with shaking at 180 rpm, the media were filtered with four layers of gauze and used for protein isolation.

**Generation of deletion mutants and complemented strains.** Approximately 1-kb flanking regions, proximal to the initiation codon and downstream of the termination codons of *bcactA, bcmn, bcmamy, bccgh, bcesg, bcga, bcabf, bccbh, bccbg, and bchca*, were individually amplified. The amplified PCR products were cloned into pLOB7 (46), verified by sequencing, and introduced into protoplasts of the wild-type B05.10. Protoplast formation and transformation were performed according to the methods of a previous study (22). Briefly, the mycelium was suspended in 20 ml of 0.5% Glucanex solution (Sigma, St. Louis, MO) and incubated at 25°C and 100 rpm for 2 h to generate protoplasts. The transformation fragments were added into a suspension of 200 ml containing 2 × 10⁷ protoplasts. Thereafter 200 μl and 400 μl of 25% polyethylene glycol (PEG) 3350 (Sigma) in 50 mM CaCl₂ and 10 mM Tris-HCl (pH 7.5) were added separately. The mixture was then transferred to SH agar, which contained 0.6 M sucrose, 5 mM HEPES, 1 mM NaCl, 1% agar, and 1% agar, to regenerate *B. cinerea* protoplasts. Transformants were initially selected on plates with hygromycin B and then checked using PCR.

Single-spor isolation was conducted to purify the deletion mutants. To verify single-copy genomic integration, Southern blot analysis was performed. Genomic DNA of wild type and three deletion mutants was digested with appropriate restriction enzyme pairs. Southern hybridization was performed with the right flank of the gene as a probe.

For complementation, the DNA fragments of specific genes, along with nucleotides approximately 2 kb upstream and 0.5 to 1 kb downstream, were amplified using wild-type genomic DNA and cloned into the pNAN-OGG vector with resistance to nourseothricin (23). The resulting plasmid was amplified, and the PCR products were used to transform the corresponding mutants. The transformants were initially selected on plates with nourseothricin and then identified by PCR. All primer pairs are listed in Table S1 in the supplemental material.

**Isolation of extracellular proteins.** Extracellular proteins were isolated according to methods described previously (21). The separated media of wild-type and ΔbcactA strains were centrifuged three times at 20,000 × g for 30 min at 4°C to remove residual mycelia or other debris. The supernatant was mixed with an equal volume of Tris-HCl (pH 7.5)-buffered phenol by shaking for 30 min at 4°C and centrifuged at 10,000 × g for 40 min to eliminate contaminants. The lower phenol phase was mixed with 5 volumes of 0.1 M ammonium acetate in methanol and incubated at −20°C overnight to precipitate the proteins. Precipitated proteins were separated by centrifugation at 15,000 × g for 30 min at 4°C. The pellet was washed twice with prechilled 0.1 M ammonium acetate in methanol and twice with prechilled acetone. Protein pellets were dried at −80°C and stored until further use.

**iTRAQ labeling and protein identification.** For secretome analysis, the extracellular proteins of WT and ΔbcactA strains were solubilized in the protein buffer, consisting of 100 mM triethylenammonium bicarbonate (TEAB) and 0.04% (wt/vol) sodium dodecyl sulfate (SDS), pH 8.5. Protein concentrations were determined using the Bradford assay (47). Sixty micrograms of proteins from each sample was reduced by adding tris-(2-carboxyethyl)phosphine (TCEP) to a final concentration of 10 mM and incubated at 60°C for 1 h. Methyl methanethiosulfonate (MMTS) was added to each sample to a final concentration of 50 mM and incubated at 25°C for 10 min. The samples were then digested with 1.2 μg trypsin (Promega) at 37°C overnight. The tryptic peptides were labeled with an iTRAQ reagent 4-plex kit (Applied Biosystems) according to the manufacturer’s instructions. The iTRAQ tags were able to mark the free amino ends. Two independent biological replications were performed. In the first biological replication, the wild-type and ΔbcactA strains were labeled with iTRAQ tags 114 and 116, respectively. In the other biological replication, the WT and ΔbcactA strains were labeled with iTRAQ tags 115 and 114, respectively. The labeled samples of each biological replication were separately pooled, vacuum dried, reconstituted in 0.1% formic acid, and submitted for nanoLC-MS/MS analysis.

The mass spectrometry (MS) analysis was performed using a nanoLC system (NanoLC-2D Ultra Plus; Eksigent, USA) coupled with a Triple TOF 5600 Plus mass spectrometer (AB ScieX) (48). The iTRAQ-labeled peptide samples were desalted with a 100-μm by 20-mm trap column and separated on an analytical 75-μm by 150-mm column packed with a Magic C₁₈ AQ 5-μm, 200-Å phase (Michrom). The mobile phase A was 0.1% formic acid in water, while the mobile phase B was 0.1% formic acid in acetonitrile. Peptides were eluted in a linear gradient of 5% to 30% mobile phase B at a flow rate of 300 nl min⁻¹. Precursor ions were selected across the mass range of 350 to 1,500 m/z⁻¹ in high-resolution mode (>30,000). A maximum of 25 precursors per cycle from each MS spectrum were selected for fragmentation with 100-ms minimum accumulation time for each precursor. Tandem mass spectra were recorded in high-sensitivity mode (resolution, >15,000).
Protein identification and quantification were performed using the ProteinPilot 4.5 software (AB Sciex). Mass tolerance for fragment ions was automatically set by the software when using the Triple TOF 5600 Plus mass spectrometer. A database search was performed against the *B. cinerea* protein database with the following parameters: (i) sample type: iTRAQ 4-plex (peptide labeled); (ii) cysteine alkylation: MMTS; (iii) digestion: trypsin; (iv) instrument: Triple TOF 5600; (v) species: none; (vi) quantitate: yes; (vii) bias correction: yes; (viii) background correction: yes; (ix) search effort: thorough; (x) FDR analysis: yes. For iTRAQ quantitation, the peptide for quantification was automatically selected by the ProGroup algorithm (AB Sciex) to calculate the reporter peak area. A reverse database search strategy was applied to estimate the global FDR for peptide identification. Only proteins identified below the 1% global FDR were ultimately exported, and a 2-fold cutoff was used as a determinant for proteins showing a significant change in abundance. A Venn diagram was generated by a web-based Venn diagram software program (http://bioinfogp.cnb.csic.es/tools/venny/index.html), and a heat map (Pearson algorithm) was constructed using the PermutMatrix software (version 1.9.3).

**Phenotype analysis.** For radial growth assays, hyphal disks of the WT and mutants with a diameter of 1 mm were removed from the growing edges. The disks were excised using a cut pipette tip (200 μl, yellow; Axygen, CA, USA) with an inner diameter of 1 mm. Cultures were grown at 22°C, and measurements were taken daily from 1 to 3 days after inoculation. For sporulation assays, spores were harvested 10 days later using distilled water, and the suspension was filtered through four layers of gauze to remove mycelial fragments. The spores were counted with a hemocytometer under a microscope.

**Virulence assays.** Spores were harvested by adding sterile water to CM cultures and filtering the mixture through four layers of gauze to remove mycelial fragments. The density of the spore suspension was determined using a hemocytometer under a microscope. Infection assays in tomato and apple fruits were performed according to the methods described in our previous study (49). Tomato and apple fruits were sterilized by immersion in 2% sodium hypochlorite solution for 2 min, rinsed with tap water, and wounded to the same depth (approximately 4 mm) with a sterile nail. Conidia were adjusted to 1 × 10⁴ spores ml⁻¹, and 10 μl was inoculated into the wounds of apples and tomatoes. Distilled water was used as negative control. Lesion formation was examined at 3 to 5 days after inoculation. For leaf infection assays, conidia were diluted to 2 × 10⁴ spores ml⁻¹ with PDB, and 10 μl was dropped onto tomato leaves harvested from 4-week-old tomato plants. The leaves were placed in moist chambers at 22°C for lesion formation. All experiments were repeated three times, and 15 apples, nine tomatoes, and six leaves were used in each replicate.

**Western blotting.** Total cytoplasmic proteins were extracted from 2-day-old mycelia cultured in PDB, according to methods described previously (29). Protein samples (20 μg) were separated by 12% SDS-PAGE and then electrotransferred onto a polyvinylidene difluoride (PVDF) membrane by semidry transfer. The membrane was blocked with 5% nonfat milk in phosphate-buffered saline with Tween 20 (PBST) buffer for 1.5 h at room temperature. Immunoblotting was conducted by incubation with a commercially available antibody of actin (Abmart; M20011), produced by immunizing mice with the human actin full-length protein. The immunoreactive bands were visualized by using a chemiluminescence detection kit (SuperSignal; Pierce Biotechnology). Histone 3 (H3) was used as a loading control.

**Statistical analysis of data.** Statistical differences in phenotype observation and virulence determination experiments were determined using the SPSS software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and Tukey’s test were used, and a *P* value of <0.05 was considered statistically significant.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, TIF file, 2 MB.

**FIG S2**, TIF file, 0.3 MB.

**FIG S3**, TIF file, 0.1 MB.

**FIG S4**, TIF file, 0.3 MB.

**FIG S5**, TIF file, 0.3 MB.

**FIG S6**, TIF file, 1 MB.

**FIG S7**, TIF file, 0.6 MB.

**FIG S8**, TIF file, 0.2 MB.

**TABLE S1**, DOCX file, 0.03 MB.

**ACKNOWLEDGMENTS**

We thank Paul Tudzynski, Julia Schumacher (Westfaelische Wilhelms-Universitaet Muenster, Germany), and Jan van Kan (Wageningen Agricultural University, The Netherlands) for kindly providing the *B. cinerea* haploid strain B05.10 and vectors.

This work was supported by The Key Program of National Natural Science Foundation of China (grants 31930086 and 31530057), The National Key R & D Program of China (2016YFD0400902), and Youth Innovation Promotion Association CAS (2018107).
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