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Involvement of BcVeA and BcVelB in regulating conidiation, pigmentation and virulence in *Botrytis cinerea*

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ABSTRACT

The heterotrimeric velvet complex VeA/VelB/LaeA is involved in the regulation of diverse cellular processes in *Aspergillus nidulans*. In this work, we investigated functions of two velvet-like genes *BcVEA* and *BcVELB* in *Botrytis cinerea*. Morphological characterization of *BcVEA* and *BcVELB* deletion mutants showed that the deletion of *BcVEA* and *BcVELB* led to increased conidiation and melanin biosynthesis. *BcVEA* and *BcVELB* deletion mutants also showed increased sensitivity to oxidative stress. Pathogenicity assays revealed that both BcVeA and BcVelB were essential for full virulence of *B. cinerea*. Yeast two-hybrid assay displayed the interaction of BcVeA with BcVelB. Results of this study indicate that BcVeA and BcVelB coordinate similar processes in the regulation of fungal development, oxidative stress response, and virulence in *B. cinerea*.

1. Introduction

Botrytis cinerea is an ascomycete responsible for gray mould on hundreds of dicotyledonous plants (Elad et al., 2004). Damages caused by *B. cinerea* are very difficult to estimate. The current global expenses of *Botrytis* control easily surmount €1 billion/annum. The impacts of product loss occurring despite disease control, and the quality loss during the retail chain, are likely to be far higher (Dean et al., 2012). Since *B. cinerea* has a variety of modes to attack its diverse hosts under a wide range of environmental conditions, and it can survive as mycelia and conidia or for extended periods as sclerotia in crop debris (Willamson et al., 2007), it is difficult to control gray mould. Thus, well understanding of fungal development and pathogenesis of *B. cinerea* will lead to the greatest impact on disease control. To date, the availability of the genome sequence and a variety of molecular tools, together with its economic relevance, have contributed to *B. cinerea* being the most extensively studied necrotrophic fungal pathogen (Amselem et al., 2011; Dean et al., 2012). Recent studies confirm that the gray mold fungus shares some conserved virulence factors with other fungal plant pathogens, but it also reveals unique features that may be linked to its polyphagous behavior (reviewed by Choquer et al., 2007).

In *Aspergillus nidulans* and several other fungal species, the heterotrimeric velvet complex VeA/VelB/LaeA has been demonstrated to play key roles in the regulation of secondary metabolism and differentiation processes such as asexual or sexual sporulation and sclerotia or fruiting body formation (reviewed by Bayram and Braus (2012)). Within this complex, VelB interacts with the N-terminal part of VeA, whereas LaeA interacts with the C-terminal part of VeA (Bayram et al., 2008). Recently, the similar interaction has also been reported from *Fusarium fujikuroi* (Wiemann et al., 2010). Among the three components, VeA was first identified in *A. nidulans* as a positive regulator of sexual reproduction and negative regulator of asexual development (Yager, 1992; Kim et al., 2002). Further studies showed that VeA was also involved in secondary metabolism as a positive regulator in *A. nidulans* (Kato et al., 2003). Soon thereafter, effects of VeA on secondary metabolism were reported from several fungi, including *Aspergillus parasiticus* (Calvo et al., 2004), *Aspergillus flavus* (Duran et al., 2007; Cary et al., 2007), *F. fujikuroi* (Wiemann et al., 2010), *Fusarium verticillioides* (Myung et al., 2009) and *Fusarium graminearum* (Merhej et al., 2012; Jiang et al., 2011). In addition to the regulation of secondary metabolism, VeA homologs have been found to be involved in various cellular processes which were not described in *Aspergillus* spp. In *F. verticillioides*, deletion of FvVE1 (the homolog of VeA) led to less aerial hyphal growth, yeast-like growth, and reduced colony surface hydrophobicity on solid media. In addition, deletion of FvVE1 increased the ratio of macroconidia to microconidia (Li et al., 2006). In *Mycosphaerella graminicola*, the VeA deletion mutant showed hypersensitivity to shaking force, which is not related to loss of hydrophobicity of cellular surface in the mutant (Choi and Goodwin, 2011). Similar to VeA, LaeA is also involved in regulating aflatoxin and sclerotial productions in *A. flavus*, but it shows distinct roles in terms of vegetative growth, conidiation, density dependent responses, and pattern of colonization of host tissues

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(Amaike and Keller, 2009). In *F. verticillioides*, the deletion of Lae1 resulted in reduced expression of gene clusters responsible for synthesis of the secondary metabolites bikaverin, fumonisins, fusaric acid and fusarins. Analysis of secondary metabolites revealed that different from what was seen in *F. fujikuroi LAE1* mutant (Wiemann et al., 2010), bikaverin production is reduced, but the amount of fumonisin is unchanged in the *F. verticillioides LAE1* mutant (Butchko et al., 2012).

In contrast to VeA and LaeA, the roles of VelB are poorly understood. A recent study on *A. nidulans* showed that asexual sporulation in VelB deletion mutant is impaired but not as strongly as that in the VeA deletion strain (Bayram et al., 2008). VelB deletion mutant exhibited reduced and delayed but significant sterigmatocystin (ST) production, whereas ST production is abolished in VeA and LaeA mutants (Bayram et al., 2008). In *A. nidulans*, VelB is not only a component of the nuclear VeA/VelB/LaeA complex, but can also form (1) a VelB homodimer in the cytoplasm as well as in the nucleus; and (2) be part of the nuclear VelB–VosA heterocomplex, which is hardly detectable in the cytoplasm (Sarikaya Bayram et al., 2010). Phenotypic analysis showed that conidia of both VelB and VosA deletion mutants displayed severe viability defects, whereas conidial viability of VeA deletion mutant was similar to that of the wild-type strain (Sarikaya Bayram et al., 2010), indicating the VelB–VosA complex plays as specific role in conferring conidial viability. Different from the scenario in *A. nidulans*, VelB executes the similar functions as does VeA in *F. fujikuroi* and *F. graminearum* (Wiemann et al., 2010; Jiang et al., 2012; Lee et al., 2012).

BLAST search of *B. cinerea* genome showed that the fungus contains two homologs of the velvet genes, *BcVEA* and *BcVELB*. Based on previous reports, we hypothesized that *BcVEA* and *BcVELB* would play important roles in the regulation of fungal development and virulence in *B. cinerea*. To address this hypothesis, the main objective of this study was to analyze functions of *BcVEA* and *BcVELB* using target gene deletion strategy.

2. Materials and methods

2.1. Strains and culture conditions

The *B. cinerea* strain 38B1 isolated from a grape in California, USA, was used as the wild-type progenitor for the transformation experiment in this study. The wild-type progenitor and its derived mutants were grown on potato dextrose agar (PDA) (200 g potato, 20 g glucose, 20 g agar, and 1 L water) for examination of colony morphology and conidiation assays.

2.2. Sequence analysis of BcVEA and BcVELB

BcVEA (BC1G_02976.1) and *BcVELB* (BC1G_11858.1) were originally identified during homology search of the *B. cinerea* genome sequence (http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html) by using BLASTP algorithm with the VeA and VelB proteins from *A. nidulans* (Bayram et al., 2008) as queries. To verify the existence and size of the introns, RNA was extracted from mycelia of the wild-type strain 38B1 with a TaKaRa RNAiso Reagent (TaKaRa Biotech. Co., Dalian, China), and was used for reverse transcription with a RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas Life Sciences, Burlington, Canada) according to the manufacturer's instructions. Reverse transcription PCR was performed with the primer pairs BcVeA-y2h-F and BcVeA-y2h-R, BcVelB-y2h-F and BcVelB-y2h-R for *BcVEA* and *BcVELB*, respectively (Supplementary Table 1). The resultant PCR products were purified, cloned and sequenced.

2.3. Construction of BcVEA and BcVELB deletion mutants

A *BcVEA* deletion vector pCA-BcVeA-UD was constructed by inserting two flanking sequences of *BcVEA* into two sides of *HPH* (hygromycin resistance) in the pBS-HPH1 vector (Liu et al., 2007). A 354-bp upstream flanking fragment of *BcVEA* amplified from 38B1 genomic DNA using the primer pair BcVeA-up-F and BcVeA-up-R was inserted into *Xho* I-*Sal* I site of the pBS-HPH1 vector to generate the plasmid pBS-BcVeA-up. Subsequently, a 710-bp downstream flanking fragment of *BcVEA* amplified from 38B1 genomic DNA using the primer pair BcVeA-down-F and BcVeA-down-R was inserted into *Bam*H I-*Sac* I site of the pBS-BcVeA-up vector to generate the plasmid pBS-BcVeA-UD. The linear DNA fragment for transformation was amplified from pBS-BcVeA-UD using the primer pair BcVeA-up-F and BcVeA-down-R, and transferred into the wild-type strain 38B1. Using the same strategy, vectors were constructed for the disruption of *BcVELB*. Protoplast formation and transformation of *B. cinerea* were performed using previously published protocols described by Schulze Gronover et al. (2001).

2.4. Complementation of BcVEA and BcVELB deletion mutants

To confirm that phenotypes of the *BcVEA* and *BcVELB* deletion mutants are due to the gene deletions, *BcVEA* deletion mutant ΔBcVeA and *BcVELB* deletion mutant ΔBcVelB were complemented with full length of *BcVEA* and *BcVELB*, respectively. The complementation plasmid SUR-BcVEA-C was constructed on the backbone of pBS-HPH1. First, the chlorimuron-ethyl resistance gene (*SUR*) was amplified from plasmid PCB1532 (Sweigard et al., 1997) with the primer pair SUR-F and SUR-R, and cloned into the *Sal* I–*Hind* III site of pBS-HPH1 to create plasmid pBS-SUR. Then, the complete *BcVEA* gene including 1669-bp upstream and 731-bp terminator region was amplified from genomic DNA of the wild-type strain with the primer pair BcVeA-com-F and BcVeA-com-R, and cloned into the *Pst* I–*Xba* I site of pBS-SUR to generate the complementation plasmid pBS-BcVeA-C. Linear DNA fragment for transformation was amplified from pBS-BcVeA-C using the primer pair SUR-F and BcVeA-com-R. *BcVELB* complementation vector pBS-BcVelB-C was constructed using the same strategy. Transformation of ΔBcVeA and ΔBcVelB was conducted as described above except that chlorimuron-ethyl was used as a selection agent.

2.5. Examination of melanin biosynthesis and sclerotial formation

To examine melanin biosynthesis, a 5-mm mycelial plug taken from the edge of a 3-day-old colony of each strain was inoculated on a PDA plate supplemented with or without tricyclazole at 50 μg/ml. After incubation at 25 °C for 9 days in the dark, melanin production was examined for each plate. The experiment was repeated three times independently.

To examine sclerotial development, a 5-mm mycelial plug taken from the edge of a 3-day-old colony of each strain was inoculated on a PDA plate. After the plates were incubated at 25 °C for 4 weeks in the dark, sclerotial formation on each PDA plate was examined. The experiment was repeated three times independently.

2.6. Determination of sensitivity of the mutants to various stresses

Mycelial growth tests under different conditions were performed on PDA supplemented with the following products: the osmotic agents NaCl, KCl, and D-sorbitol; oxidative stress generators H_2O_2 and paraquat; cell wall damaging agents, caffeine, Congo red and SDS; and the antifungal drug rapamycin at various concentrations. Each plate was inoculated with a 5-mm diameter mycelial plug taken from the edge of a 3-day-old colony grown on PDA. After the plates were incubated at 25 °C for 2 days, colony diameter