Coordinated and independent functions of *velvet*-complex genes in fungal development and virulence of the fungal cereal pathogen *Cochliobolus sativus*

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Abstract

LaeA and *velvet* proteins regulate fungal development and secondary metabolism through formation of multimeric complexes in many fungal species, but their functions in the cereal fungal pathogen *Cochliobolus sativus* are not well understood. In this study, four *C. sativus* genes (*CsLaeA*, *CsVeA*, *CsVelB*, and *CsVelC*), which are orthologous to four components (*LaeA*, *VeA*, *VelB*, and *VelC*) of the *velvet* complexes, respectively, were identified, and knockout mutants for each of the genes were generated and characterized. Both $\Delta CsVeA$ and $\Delta CsVelB$ showed significant reduction in aerial mycelia growth. $\Delta CsVelB$ also exhibited a hypermorphic conidiation phenotype with indeterminate growth of the conidial tip cells and premature germination of conidia. $\Delta CsLaeA$, $\Delta CsVeA$, and $\Delta CsVelB$ produced more conidia under constant light conditions than under constant dark conditions whereas no differences were observed under the two conditions for the wild type. Under constant light conditions, the three knockout mutants ($\Delta CsLaeA$, $\Delta CsVeA$, and $\Delta CsVelB$) showed significantly reduced conidiation compared to the wild type; they also produced more small sized conidia, especially under the constant dark conditions. These mutants had less conidial pigmentation probably due to reduced expression of genes involved in melanin production as revealed by RNA expression assay for *PKS18*, one of the major genes involved in the melanin biosynthesis pathway in *C. sativus*. All knockout mutants ($\Delta CsLaeA$, $\Delta CsVeA$, $\Delta CsVelB$ and $\Delta CsVelC$) showed some extent of reduction in virulence on susceptible barley plants compared to the wild type strain. The results revealed the conserved and unique roles of

velvet-complex proteins as regulators in mediating fungal development and secondary metabolism in *C. sativus*.

Introduction

Spot blotch, caused by the ascomycete fungus *Cochliobolus sativus* (Ito & Kurib.) Drechs. ex Dastur. [anamorph: *Bipolaris sorokiniana* (Sacc.) Shoem.], is one of the most common and economically important diseases in barley. It can cause more than 30% yield loss and significantly impact the malting quality of barley (Wilcoxson et al. 1990). *C. sativus* is a polycyclic plant pathogen and the disease cycle begins when conidia (asexual spores) land on the leaf surface of barley or wheat. Subsequently, the conidium germinates and develops an appressorium, a specialized infection structure, which assists in penetration into the leaves (Kumar et al. 2002). After successful penetration, the fungus produces infection hyphae to invade into the leaf mesophyll tissues of the host (Santen et al. 2005). Then, necrosis and/or chlorosis appears in infected host cells and their neighboring non-infected cells, indicating that certain toxic compounds (virulence factor) released by the fungus diffuse into non-infected host cells (Kumar et al. 2002). Previous studies with pathotype 2 of *C. sativus* (ND90Pr) on the barley cv. Bowman have revealed a secondary metabolite peptide as the virulence factor synthesized by non-ribosomal peptide synthetases (NRPSs) (Condon et al. 2013). Based on the pathogen's life cycle and its infection process, the incidence and severity of the spot blotch disease are closely associated with (1) repetitive rounds of new infections; (2) the amounts of conidia produced during each round of conidiation; and (3) the productivity of virulence factor. Therefore, understanding the molecular regulation of conidiation and secondary metabolite

biosynthesis in this fungus is a prerequisite for developing sustainable disease management strategies.

Recent studies revealed that LaeA and *velvet* proteins (VeA, VelB, VelC and VosA) act as global regulators of fungal development and secondary metabolism through formation of multimeric complexes, such as LaeA/VeA/VelB, VelB/VosA, VelB/VelB (Bayram et al. 2008; Bayram et al. 2010; Park et al. 2012a), VelC/VosA (Park et al. 2014), and VelC/VeA (Lan et al. 2014). These *velvet*-complex proteins are highly conserved in many fungal species and have been extensively studied in a number of fungi. LaeA was identified as a putative methyltransferase in *Aspergillus nidulans* (Bok and Keller 2004) and was reported to positively regulate the asexual development of *A. nidulans*, *Penicillium chrysogenum*, and *Fusarium fujikuroi* (Bayram et al. 2010; Kosalkova et al. 2009; Wiemann et al. 2010). In *Cochliobolus heterostrophus*, however, deletion of ChLae1 increases asexual sporulation and female sterility, reduces tolerance to oxidative stress and aerial hyphal growth (Wu et al. 2012). The other four components of *velvet*-complex are *velvet* superfamily proteins, which have been shown to be regulators in several fungal species by acting as transcription factors (Ahmed et al. 2013). VeA was first identified as a positive regulator of sexual sporulation and negative regulator of asexual development in *A. nidulans* (Kafer 1965). Similarly, in *C. heterostrophus*, ChVel1 negatively controls asexual differentiation, but positively regulates oxidative stress responses, sexual development, and aerial hyphal growth (Wu et al. 2012). Furthermore, in *Fusarium verticillioides*, deletion of FvVE1 suppresses aerial hyphal growth, reduces colony

surface hydrophobicity, and activates conidiation with an increased ratio of macroconidia to microconidia (Li et al. 2006). In contrast, the VeA homologs in *Aspergillus flavus*, *P. chrysogenum* and *Fusarium graminearum* positively regulate asexual reproduction (Amaike and Keller 2009; Hoff et al. 2010; Merhej et al. 2012). VelB and VosA forms VelB/VosA and VelB/VelB dimers, positively controlling trehalose biosynthesis, spore viability and conidial germination but negatively controlling the conidial production in *A. nidulans*, *C. heterostrophus* and *C. sativus* (Ni and Yu 2007; Park et al. 2012b; Wang et al. 2015; Wang et al. 2014). In rice blast fungus *Magnaporthe oryzae*, *MoVLEB* positively regulates the conidial germination and conidia production, whereas *MoVOSA* appears dispensable for the fungal development (Kim et al. 2014). VelC was studied only in a few fungal species. Deletion of VelC in *A. nidulans* increases conidiation but significantly reduces the number of sexual fruiting bodies (Park et al. 2014). Similarly, deletion of PcVelC in *P. chrysogenum* also increases conidiation (Kopke et al. 2013). In contrast, deletion of *MoVELC* in *M. oryzae* affects the cell wall integrity of appressoria, significantly decreases the conidia production, and reduces the conidial size (Kim et al. 2014).

LaeA and the *velvet* proteins also play important roles in the regulation of secondary metabolism. For example, deletion of LaeA in *A. nidulans* blocks the expression of metabolic gene clusters, leading to reduced production of sterigmatocystin, penicillin, and lovastatin (Bok and Keller 2004). The VeA orthologs had been reported to control biosynthesis of various secondary metabolites such as aflatoxin, cyclopiazonic acid, and aflatrem in *A. flavus*

(Duran et al. 2009), trichothecenes in *F. graminearum* (Merhej et al. 2012), and melanin in *Mycosphaerella graminicola* (Choi and Goodwin 2011). In *F. verticillioides*, Fvve1 and Fvve1B positively regulate fumonisin production (Lan et al. 2014). In *P. chrysogenum*, PcLaeA, PcVelA, and PcVelC activate penicillin biosynthesis, whereas PcVelB represses this process. In *C. heterostrophus*, ChLae1 and ChVel1 positively regulate T-toxin biosynthesis but negatively control melanin biosynthesis in mycelia (Wu et al. 2012). In the fungal cereal pathogen *C. sativus*, CsVosA plays a positive and negative role in melanin production in conidia and mycelia, respectively, and positively regulates the biosynthesis of virulence related secondary metabolites (Wang et al. 2015).

All these studies indicate that although *velvet*-complex genes are conserved among many filamentous fungi, their functions on regulating fungal development and biosynthesis of secondary metabolites may be similar or different depending on the fungal species involved. In this study, we identified and characterized *CsLaeA*, *CsVeA*, *CsVelB* and *CsVelC* in *C. sativus* and showed their functions in regulating oxidative stress tolerance, aerial hyphal growth, asexual development, as well as production of melanin and virulence factors. By studying the single and double gene knockout mutants, we further explored the coordinated and distinct functions of these genes in fungal development and secondary metabolism and provided a comparative framework between the functions of *velvet*-complex genes in different fungal species.

Materials and methods

Fungal isolates, media and general growth conditions

In this study, the *C. sativus* isolate ND90Pr (ATCC 201652) was used as the wild type (WT).

Unless mentioned otherwise, all isolates were grown on V8 potato dextrose agar (V8 PDA) under 12 hour fluorescent light each day at approximately 23 °C. Potato dextrose broth (PDB), minimal medium (MM) (Tinline et al. 1960), and water agar plates were also used for fungal growth in specific experiments.

Identification of the LaeA, VeA, VelB and VelC orthologs in *C. sativus*

The *A. nidulans* LaeA, VeA, VelB and VelC (accession number: CBF88745.1, AAD42946.1, ABQ17967.1, and ABQ17968.1) protein sequences were used to query the genome sequence of the *C. sativus* isolate ND90Pr (<http://genome.jgi-psf.org/Cocsa1/Cocsa1.home.html>) using the BLASTP algorithm. The protein sequences of CsLaeA, CsVeA, CsVelB and CsVelC and their corresponding gene sequences can be found in Energy Joint Genome Institute (<http://genome.jgi-psf.org/Cocsa1/Cocsa1.home.html>) under the protein ID 175491, 101845, 173198, and 119375, respectively. cNLS Mapper program (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) was used to predict the putative nuclear localization signal.

Gene replacement and complementation

Single or double *velvet*-complex genes were deleted and replaced by a fragment carrying a hygromycin phosphotransferase gene (*hph*) or/and a fragment carrying the neomycin phosphotransferase II gene (*nptII*) using the split-marker system (Supplemental Fig. 1) (Catlett et al. 2003). The transformation protocol was the same as previously described by (Leng and Zhong 2012). Complementation was conducted with two methods. For complementation of $\Delta CsLaeA$ and $\Delta CsVeA$, the coding region of the corresponding gene was cloned into the pSGATE1-G418-2 vector carrying the *nptII* gene for neomycin resistance (Leng et al. unpublished) using the Gateway cloning system. The constructed vectors containing *CsLaeA* or *CsVeA* gene were linearized by BstXI and SacI digestion, respectively, and transformed into the corresponding single knockout mutant ($\Delta CsLaeA9$ and $\Delta CsVeA9$). For complementation of $\Delta CsVelB$ and $\Delta CsVelC$, a co-transformation method was applied. First, the coding region and flanking sequences containing promoter of the corresponding gene were cloned into the pGEM®-T Easy vector. Second, the constructed T Easy vector and the plasmid G418 carrying the *nptII* gene for neomycin resistance were linearized by NdeI and *EcoRI* digestion, respectively, and then were co-transformed into the single knockout mutant ($\Delta CsVelB3$ and $\Delta CsVelC2$). The primers used for gene deletion and complementation are listed in Supplemental Table 1. The procedure for Southern blot and PCR confirmation was the same as previously described in Leng and Zhong (2012). The enzymes used for digestion of the wild type genomic DNA were indicated in Supplemental Fig. 1. Sequences of primers for hybridization probe and PCR confirmation are listed in Supplemental Table 1.

Assays of oxidative stresses

To evaluate the sensitivity of conidia to oxidative stress, conidia of each fungal strain grown on V8 PDA for 6 days were harvested and plated on MM plates supplemented with different concentrations (0, 1.3 and 2.6 mM) of H₂O₂, and then incubated at 25 °C for 12 hours before the conidial viability rate was calculated. The conidial viability was calculated as the percentage of viable conidia in the total number of conidia examined. Only those conidia producing hyphae at least 5 times longer than the conidia at 12 hours post-incubation were considered viable since the *CsVelB* mutant conidia germinated when they were attached to the conidiophores but they failed to grow on medium plates. Three replicates were used for each fungal strain and 100 conidia were examined for each replicate.

To evaluate the sensitivity of mycelium to oxidative stress, a small mycelial plug (1 mm × 1 mm) of each fungal strain grown on V8 PDA for 3 days was cut and placed on the center of V8 PDA supplemented with H₂O₂ at final concentrations of 0, 4, 6, and 8 mM, respectively. After 7 days of incubation at room temperature, the radial diameter of the fungal colony was measured. Three replicates were used for each strain.

Analysis of conidial productivity, conidiation pattern and conidiophore production

To measure conidial productivity, single conidium of wild type, knockout mutants and the complemented strains were inoculated on the center of V8 PDA plates and allowed to grow for 7 days at 25 °C under constant dark or constant light conditions. Conidia were harvested

and quantified as described by Leng and Zhong (2012). The experiments were performed in triplicates.

To investigate the pattern of conidial formation, the conidia were inoculated on the slide culture with MM. After 5 days of growth, conidiation patterns were examined and photographs were taken using Olympus BX51 microscope (Olympus, Center Valley, PA, USA) with the CCD camera digital imaging system (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) under the 20X magnification.

To measure the conidiophore production, single conidium of wild type and knockout mutant was inoculated on the center of V8 PDA plates and allowed to grow for 7 days at 25 °C under constant dark or constant light conditions, respectively. The number of conidiophores per square centimeter of medium plates (only the conidiation sections were considered for $\Delta CsVeA9$ and $\Delta CsVelB3$) was counted with a dissecting microscope. The conidiophore production of wild type grown under constant dark conditions was used as a reference to calculate the conidiophore productivity rate.

Examination of conidial size and pigmentation

To examine the size and pigmentation of conidia, wild type and mutants were grown on V8 PDA under constant dark or constant light conditions for 7 days. Conidia of each strain were harvested and photographed using an Olympus BX51 microscope (Olympus, Center Valley,

PA, USA) with the CCD camera digital imaging system (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) under 20X magnification. The average conidial size was calculated based on the area of conidia on the pictures that were measured by the software SigmaScan Pro 5 (Systat Software Inc., San Jose, CA, USA). The average pixel brightness intensities (BI) of the conidia on the pictures were also measured using the SigmaScan Pro 5 software. The pigment intensity (PI) was converted from the average pixel brightness intensity using $PI = 1/BI$.

Examination of spore viability and appressorial formation on barley leaves

To investigate spore viability and appressorial formation on barley leaves, conidia of the wild type, knockout mutants and complemented strains grown on V8 PDA at 25 °C for seven days were harvested and spray inoculated on 10~12 days old seedlings of barley cv. Bowman (1 mL per plant at 5×10^3 conidia mL⁻¹). The inoculated plants were misted with distilled water and then covered with a black plastic bag to maintain high humidity. After 12 hours of incubation under dark conditions at room temperature ($23 \pm 2^\circ\text{C}$), barley leaves were sampled and examined for spore viability under a compound microscope. The spore viability was calculated as the percentage of viable conidia in the total number of conidia examined. Only those conidia producing hyphae at least 5 times longer than the conidia after 12 hours of incubation on barley leaves were considered viable since the *CsVelB* mutant conidia germinated when they were attached to the conidiophores but they failed to grow on barley leaves. Appressorial formation was examined on leaf segments sampled at 12 and 24 hours

after inoculation (HAI) and subjected to treatments according to the method as previously described (Wang et al. 2015). Three replicates (barley leaves) were used for each fungal strain and 100 conidia were examined for each replicate.

Pathogenicity test

Pathogenicity tests were performed by spray inoculation with conidial suspension on 10~12 days old seedlings of barley (cv. Bowman) plants (when the second leaves were fully expended) according to Fetch and Steffenson (1999), except the concentration of the conidial suspension was at 1.5×10^3 conidia/mL. Inoculated plants were incubated in a humidity chamber for 20-24 hours, and then transferred into a greenhouse (23 ± 2 °C) and incubated for 7 days before the spot blotch disease was rated using the 1-9 rating scale of Fetch and Steffenson (1999). For each fungal strain, at least nine plants were used for inoculation and the experiment was repeated three times. The photographs of infected leaves were taken using digital camera (Nikon D80 with lens Af-S Micro NIKKOR 60 mm).

RNA extraction and gene expression analyses

For expression analyses of *PKS18* (Leng et al. 2012), total RNA was extracted from conidia harvested from the culture of each strain grown on V8 PDA under constant dark for 7 days.

For expression analyses of the NRPS encoding genes (protein IDs 115356 and 140513), RNA was extracted from a mixture of mycelia harvested from the culture at 96 HAI (hours after inoculation) from different media including PDA, MM, V8 PDA, and water agar (sample

M96) as well as from inoculated barley leaves at 12, 24, and 48 HAI on bowman (sample 12HAI, 24HAI, and 48HAI). RNA was extracted using SV Total RNA Isolation System (Promega, Madison, WI) and purified by treatment with DNase I (New England Biolabs, Ipswich, MA). cDNA generation and RT-PCR procedure was the same as previously described in Leng and Zhong (2012). The β -actin-encoding gene (ACT) was amplified with the RT-Actin-F and RT-Actin-R primers (Supplemental Table 1) and used as reference in all RNA expression experiments. The primer pairs for each gene used in each analysis are listed in Supplemental Table 1.

Results

Identification of the *LaeA*, *VeA*, *VelB* and *VelC* orthologs in *C. sativus*

The candidate orthologs were identified from the draft genome sequence of *C. sativus* (<http://genome.jgi-psf.org/Cocsa1/Cocsa1.home.html>) using the BLASTP algorithm with *LaeA*, *VeA*, *VelB* and *VelC* of *A. nidulans* as queries. Based on the highest scores and the lowest E values, four protein orthologs were identified and designated as *CsLaeA*, *CsVeA*, *CsVelB* and *CsVelC*, respectively. The *CsLaeA* gene contains 1,445 base pairs with 7 introns and encodes a protein (368 amino acids) with a highly conserved methyltransferase domain, a characteristic of the *LaeA* genes, as identified using Pfam (<http://pfam.xfam.org/search>) (Fig. 1). The other three genes (*CsVeA*, *CsVelB* and *CsVelC*) contain 1,800, 1,089, and 1,476 base pairs, respectively, and one intron exists in *CsVelC*. The *velvet* domain was identified in all three proteins using Pfam (Fig. 1). For *CsVeA* and *CsVelC*, a putative monopartite nuclear

localization signal (NLS) and bipartite nuclear localization signal (NLS) was identified, respectively, using the cNLS Mapper program (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) (Fig. 1)

Generation of gene knockout mutants and complemented strains

Knockout mutants were generated for all four genes (*CsLaeA*, *CsVeA*, *CsVelB* and *CsVelC*) and at least three independent deletion mutants as well as one ectopic strain were generated for each of them. By introducing the *velvet*-complex gene back into the corresponding deletion mutant, at least three complemented strains were obtained for each gene. These knockout mutants and complemented strains were confirmed by PCR amplification, DNA sequencing and Southern blot analyses. Since the independent mutants generated for the same gene performed similarly, only one representative mutant for each gene was presented here. $\Delta CsLaeA9$, $\Delta CsVeA9$, $\Delta CsVelB3$ and $\Delta CsVelC2$ were single gene knockout mutants with the corresponding gene deleted, respectively; $\Delta CsLaeAVeA2$ was a double gene knockout mutant with both *CsLaeA* and *CsVeA* deleted; $\Delta CsLaeAVelB3$ was a double gene knockout mutant with both *CsLaeA* and *CsVelB* deleted; and $\Delta CsVelBVeA6$ was a double knockout mutant with both *CsVeA* and *CsVelB* deleted. As shown in Supplemental Fig. 1 and Supplemental Fig. 2A, an amplicon was only present in the wild type (ND90Pr) and complemented strains but not in the knockout mutants when the *Csvelvet*-specific primer pair SP-*Csvelvet*-F and SP-*Csvelvet*-R were used for PCR amplification. Using the primer *Csvelvet*-F0 at the flanking region of *Csvelvet* genes and the primer HYG2-R or nptIIR at the hygromycin resistance

gene (*hph*) or the neomycin resistance gene (*nptII*) for PCR, the fragments were only amplified from the knockout mutants but not from the wild type (Supplemental Fig. 2B), indicating that *Csvelvet* genes were replaced by *hph* in the single knockout mutants and also by *nptII* in the double gene knockout mutants. The results were confirmed by DNA sequencing of the PCR products (data not shown). The deletion of *Csvelvet* genes were further confirmed by Southern blot of the enzyme-digested genomic DNA from the wild type and mutants using the probes amplified from the *Csvelvet* gene encoding sequences using primer pair SP-*Csvelvet*-F and SP-*Csvelvet*-R (Supplemental Table 1). As indicated in Supplemental Fig. 1E-F, the fragments in the wild type strain and complemented strain were detected while no hybridization signals were observed in the knockout mutants.

Morphology and aerial mycelium growth of mutants on V8 PDA

Under the dark conditions, $\Delta CsVeA9$ and $\Delta CsVelB3$ as well as their derived double knockout mutants ($\Delta CsLaeAVeA2$, $\Delta CsLaeAVelB3$, and $\Delta CsVelBVeA6$) showed restricted mycelia growth compared with WT and other mutants on V8 PDA (Fig. 2A). Only some areas (conidiation sections) of the $\Delta CsVeA9$ and $\Delta CsVelB3$ cultures produced conidia under the constant dark conditions (Fig. 2A). In addition, the WT, $\Delta CsLaeA9$ and $\Delta CsVelC2$ developed long, thick, white aerial mycelia under the constant dark or light conditions, whereas $\Delta CsVeA9$ and $\Delta CsVelB3$ as well as their derived double knockout mutants showed significant reduction in the amount of aerial mycelia produced, leading to a flat surface of the

mutants (Fig. 2A&B). The complemented mutants ($\Delta CsVeAC2$ and $\Delta CsVelBC1$) with the corresponding genes (*CsVeA* and *CsVelB*) restored the WT phenotype (Supplemental Fig. 3).

Velvet-complex genes regulate responses to oxidative stress

Except for the *CsVelC* knockout mutants, the conidia of other *velvet*-complex gene knockout mutants exhibited different levels of reduction in tolerance to H_2O_2 stress than those of WT (Fig. 3A). The conidia of *CsVelB* knockout mutants ($\Delta CsVelB3$, $\Delta CsLaeAVelB3$ and $\Delta CsVelBVeA6$) were more sensitive to H_2O_2 stress than those of *CsLaeA* and *CsVeA* mutants ($\Delta CsLaeA9$, $\Delta CsVeA9$ and $\Delta CsLaeAVeA2$). We also investigated the sensitivity of mycelia to oxidative stress. As indicated in Fig. 3B, the mycelia of *velvet*-complex knockout mutants (except $\Delta CsVelC$ mutants) exhibited slower growth at the same concentration of H_2O_2 compared to the WT. However, in contrast to conidia, the mycelia of *CsLaeA* mutants ($\Delta CsLaeA9$, $\Delta CsLaeAVeA2$ and $\Delta CsLaeAVelB3$) were more sensitive to oxidative stress than those of *CsVeA* and *CsVelB* mutants ($\Delta CsVeA9$, $\Delta CsVelB3$ and $\Delta CsVelBVeA6$). The *CsLaeA* mutants were completely inhibited for growth on PDA with 6mM of H_2O_2 whereas the *CsVeA* and *CsVelB* mutants failed to grow on PDA with 8mM of H_2O_2 (Fig. 3B).

Velvet-complex genes regulate conidia production and conidiogenesis pattern

Under constant dark conditions, $\Delta CsVeA9$ and $\Delta CsVelB3$ as well as their derived double knockout mutants ($\Delta CsLaeAVeA2$, $\Delta CsLaeAVelB3$, and $\Delta CsVelBVeA6$) showed a significant increase in conidial production (Fig. 4A), whereas $\Delta CsLaeA$ and $\Delta CsVelC$ produced similar

numbers of conidia compared to WT. In contrast, under constant light conditions, $\Delta CsLaeA9$, $\Delta CsVeA9$ and $\Delta CsVelB3$ were significantly decreased in conidial production. The derived double knockout mutants ($\Delta CsLaeAVeA2$, $\Delta CsLaeAVelB3$, and $\Delta CsVelBVeA6$) produced similar amount of conidia as the *CsLaeA* mutant. $\Delta CsVelC2$ produced similar amount of conidia just like WT (Fig. 4A). In addition, all mutants except $\Delta CsVelC2$ produced more conidia under constant dark conditions than under constant light conditions (Fig. 4A).

Further examination of conidiogenesis indicated that significant differences in conidiophore production were observed between wild type and the mutants under the constant dark and light conditions (Table 1). Especially for the *CsVeA* and *CsVelB* mutants, the conidiophore production was significantly increased under constant dark conditions and significantly decreased under constant light conditions. More interestingly, the *CsVelB* mutant exhibited conidiation pattern different from WT. The wild type and other mutants ($\Delta CsLaeA9$, $\Delta CsVeA9$ and $\Delta CsVelC2$) produced 3-5 conidia on a conidiophore in a sympodial fashion, while $\Delta CsVelB3$ had conidiophores with multiple chains of conidia with the oldest conidium at the base and the youngest one emerging at the tip of the spore chain (Fig. 4B).

Velvet-complex genes regulate conidial morphology

The conidia of mutants (except $\Delta CsVelC2$) differed from those of WT in size, shape, septa production and compartmentalization (Fig. 5). The wild type strain (ND90Pr) and *CsVelC* mutant produced normal sized conidia measured at $15-28 \times 80-120 \mu\text{m}$ (area value

3,000~4,500 in SigmaScan Pro 5 software) with 4-8 septa, and having very few small sized conidia measured at $7-14 \times 15-30 \mu\text{m}$ (area value 500~1,500 in SigmaScan Pro 5 software) with 0-1 septa under constant dark and constant light conditions (Table 2). However, the knockout mutants of *CsLaeA*, *CsVeA*, and *CsVelB* produced many more small sized conidia under constant dark and/or constant light conditions, compared to WT (Table 2). $\Delta CsVelB3$ also produced some large conidia measured at $20-30 \times 120-200 \mu\text{m}$ (area value 8,000~10,000 in SigmaScan Pro 5 software), but only under constant light conditions (data not shown).

The WT conidia of *C. sativus* would not germinate when they were attached to the conidiophores. However, conidia of $\Delta CsVelB3$ and its derived double knockout mutants ($\Delta CsLaeAVelB3$ and $\Delta CsVelBVeA6$) germinated even when they were still attached to the conidiophores (Fig. 5). This indicates that *CsVelB* is involved in regulating spore dormancy.

Velvet-complex genes regulate pigmentation

As showed in Fig. 6A, all single gene knockout mutants except for $\Delta CsVelC2$ showed different levels of reduction in pigmentation, with $\Delta CsVelB3$ and its derived double knockout mutants ($\Delta CsLaeAVelB3$ and $\Delta CsVelBVeA6$) showing the highest levels of reduction.

$\Delta CsVeA9$ and double knockout mutant $\Delta CsLaeAVeA2$ had a similar level of reduction in pigmentation. To investigate if the *velvet*-complex genes affect the expression of *PKS18*, the gene involved in biosynthesis of melanin in *C. sativus* (Leng et al. 2011), mRNA levels of *PKS18* in WT and five single knockout mutants were examined using quantitative real time

PCR. The results showed that *PKS18* expression was down regulated in the mutant conidia (Fig. 6B), consistent with the pigment intensity levels measured by SigmaScan Pro 5 (Fig. 6A).

Roles of *velvet*-complex genes in pathogenicity and virulence

WT and all mutants produced spot blotch symptoms on Bowman (Fig. 7A). However, the virulence of $\Delta CsVeA9$ and $\Delta CsVelB3$ was significantly reduced while the virulence of $\Delta CsLaeA9$ and $\Delta CsVelC2$ was slightly reduced as compared to the wild type. The virulence of the double knockout mutants ($\Delta CsLaeAVeA2$ and $\Delta CsLaeAVelB3$) were slightly lower than or similar to the virulence observed for the single gene knockout mutants ($\Delta CsVeA9$ and $\Delta CsVelB3$) while the double knockout mutant $\Delta CsVelBVeA6$ was the lowest in virulence among all gene knockout mutants (Fig. 7A and B). The complemented strains for each of single gene knockout mutants restored the virulence to WT (data not shown).

To determine which stage of the disease development is affected by *velvet*-complex genes, the viability of WT and mutants conidia inoculated on leaves of Bowman were examined under a microscope. The results showed that the viability of conidia from $\Delta CsVelB3$ and its derived double knockout mutants was significantly lower than conidia of WT while no significant differences were observed between WT and other mutants along with the complemented strain for $\Delta CsVelB3$ on barley leaves (Fig. 7C). However, all mutants produced appressoria on Bowman leaves at a similar rate like the wild type (Fig. 7D).

To investigate whether the *velvet*-complex regulates the two *NRPS* genes (protein IDs 115356 and 140513) required for high virulence of isolate ND90Pr on Bowman (Condon et al. 2013), their mRNA expression levels were measured in WT and the *velvet*-complex single gene knockout mutants at different time points during host infection. The results indicated that the two *NRPS* genes had the lowest levels of expression in $\Delta CsVeA9$ and $\Delta CsVelB3$ whereas their expression levels in $\Delta CsLaeA9$ and $\Delta CsVelC2$ were only slightly reduced (Fig. 8A and B), consistent with the disease ratings.

Discussion

The LaeA protein and *velvet* proteins are highly conserved among various fungi and they have been identified in many plant fungal pathogens (Bayram and Braus 2012). In a previous study, we characterized the *VosA* gene (*CsVosA*) in *C. sativus* (Wang et al. 2015). In this study, we characterized the functions of other components of the complex (*CsLaeA*, *CsVeA*, *CsVelB* and *CsVelC*) in this fungus. *CsLaeA* contains the methyltransferase domain while the others have the *velvet* domain, indicating they are candidate orthologs of the LaeA and *velvet* proteins. The nuclear localization signals (NLSs) identified in *CsVeA* and *CsVelC* support their possible functions as transcription factors (Ahmed et al. 2013). Although the NLS was not identified in *CsVelB*, the transportation of *CsVelB* into nuclei may depend on the formation of heterodimers with *CsVeA* protein, as indicated in *A. nidulans* (Bayram et al. 2008).

Previous studies showed that *LaeA*, *VeA*, and *VelB* were positively involved in regulating the growth of aerial mycelia. For example, no or few aerial mycelial growth was observed on the cultural surface of the *VeA* knockout mutant of *M. graminicola* (Choi and Goodwin 2011), the *LaeA* and *VeA* mutants of *C. heterostrophus* (Wu et al. 2012) and *P. citrinum* (Baba et al. 2012), the *LaeA*, *VeA* and *VelB* knockout mutants of *F. oxysporum* (Lopez-Berges et al. 2013), and the *VeA* and *VelB* mutants of *F. graminearum* (Jiang et al. 2011; Lee et al. 2012). Similarly, we showed that deletion of *CsVeA* and *CsVelB* led to abolishment of the aerial mycelia growth under both constant dark and light conditions. However, the *CsLaeA* and *CsVelC* knockout mutants produced the similar amount of aerial mycelia as the wild type, suggesting they are not involved in this process in *C. sativus*. In addition, the phenotypes of the *LaeA* plus *VeA* or *VelB* double knockout mutants ($\Delta CsLaeA VeA$ or $\Delta CsLaeA VelB$) were the same as the $\Delta CsVeA$ or $\Delta CsVelB$, suggesting *CsVeA* and *CsVelB* are epistatic to *CsLaeA* in regulation of aerial mycelial growth.

Reactive oxygen species (ROS) play key roles in plant-pathogen interactions and thus we tested the sensitivities of each knockout mutant to oxidative stress at both conidia and mycelia stage. The results showed that the conidia and mycelia of $\Delta CsLaeA$, $\Delta CsVeA$, and $\Delta CsVelB$ were hypersensitive to oxidative stress mediated by H_2O_2 , indicating these genes are required to provide the protection from oxidative damage by ROS. Interestingly, the major genes involved in responses to the oxidative stress are different in conidia and mycelia.

Based on the results shown in Fig. 3, *CsVelB* mainly regulates the sensitivity to oxidative stress in conidia while *CsLaeA* is mainly involved in mycelial responses to the oxidative stress. Although *CsVeA* is involved in this process, it plays less important role as compared to *CsVelB* in conidia and *CsLaeA* in mycelia. The molecular mechanisms underlying these changes and differences are unknown in *C. sativus*. In *C. heterostrophus*, the expression of CAT3, one of three catalases in the genome central to decomposition of H₂O₂, was regulated by *lae1* (*ChLaeA*), *vel1* (*ChVeA*), *vel2* (*ChVelB*), and *vos1* (*ChVosA*) genes (Wu et al. 2012). The *velvet*-complex genes in *C. sativus* may have similar functions in controlling the expression of CAT3, but further studies are needed.

The *velvet*-complex genes negatively or positively regulate conidia production in various fungal species. For example, *VeA* in *A. nidulans* negatively regulated conidiation whereas *VelB* positively regulated this process in *A. nidulans* (Kim et al. 2002; Park et al. 2012b). In addition, *FgVel1* and *FfVel1* positively regulate conidiation in *F. graminearum* (Merhej et al. 2012) and *F. fujikuroi* (Wiemann et al. 2010), respectively. In *M. oryzae*, *MoVEA*, *MoVELB* and *MoVELC* positively regulate conidia production (Kim et al. 2014). In *P. chrysogenum*, *velvet*-complex genes are known to play functionally opposite roles in conidiation: *PcVelA* and *PcVelC* inhibits conidiation while *PcLaeA*, *PcVelB* and *PcVosA* promote conidiation (Kopke et al. 2013). In *C. sativus*, *CsVosA* was found to suppress conidia production (Wang et al. 2015). In this study, we showed that *CsLaeA*, *CsVeA*, and *CsVelB* positively regulated conidial production under light conditions. However, interestingly, under dark conditions,

CsVeA, and *CsVelB* negatively regulated conidia production. To our knowledge, this is the first report to show that the same *velvet* gene plays opposing roles under different light conditions in the same fungus.

Light plays important role in balancing between asexual and other differentiation processes in various fungi. In *A. nidulans*, sexual development is induced under dark conditions and repressed under light conditions, while asexual sporulation is regulated in a contrasting way in response to a light/dark signal (Bayram et al. 2008; Bayram et al. 2010). In addition, the differentiation processes in *Botrytis cinerea* are strictly regulated by the light regime: dark conditions promote sclerotial development while light induces conidiation (Schumacher et al. 2012). Similar light regulations were observed in *C. heterostrophus* and *P. chrysogenum*: these two fungi produce much more conidia under light than under dark conditions, indicating light promotes the conidiation (Wu et al. 2012; Kopke et al. 2013). The molecular mechanism underlying this light regulation can be partly explained by the regulatory function of VeA in these fungi. In *A. nidulans*, VeA is transferred to the nucleus only under the dark to turn on genes involved in sexual development. Deletion of VeA suppresses the sexual development and promotes the asexual development (Bayram et al. 2008; Bayram et al. 2010). In *B. cinerea*, deletion of the VeA uncouples the developmental program (sclerotial development and conidiation) from this light control, making the *BcVeA* mutant produce more conidia than wild type under both light and dark conditions (Yang et al. 2013). Similarly, deletion of VeA and VelB in *C. heterostrophus* produced more conidia than wild

type under dark and light conditions (Wu et al. 2012). For *C. sativus*, the light regulation through VeA in wild type was absent because the wild type produces the same amount of conidia under both dark and light conditions. However, deletion of VeA induces the light response and leads to production of significantly different numbers of conidia under dark and light conditions. We speculate that there is another protein that can sense the lights and regulate conidiation in *C. sativus* but is suppressed by VeA in the wild type. When VeA was deleted, this protein was activated to sense the light and regulate the conidiation, resulting in differences in conidial production under different light conditions. However, the exact molecular mechanism is not known and more studies are needed to better understand the conidiation process and the light regulation in this fungus.

In *Cochliobolus* species, conidiogenesis follows a sympodial fashion in which 3 to 5 conidia are normally produced by an expansion and swelling of the apex of the conidiophore. In this pattern, all produced conidia were attached to the same conidiophore. This pattern was observed in wild type and the three mutants, $\Delta CsLaeA9$, $\Delta CsVeA9$ and $\Delta CsVelC2$. However, both sympodial and acropetal conidiation patterns were observed in *CsVelB* knockout mutant, like the *CsVosA* knockout mutant (Wang et al. 2015), in which the new conidia were produced on the tips of older conidia, formed on the apex of the conidiophore. These results suggest that *CsVelB* and *CsVosA* suppress the acropetal conidiogenesis pattern in *C. sativus* whereas the other *velvet*-complex genes are independent for this process.

Previous studies showed that *velvet*-complex proteins regulate melanin production in various fungi. For example, *LaeA* in *C. heterostrophus* represses the production of mycelial melanin (Wu et al. 2012). *VeA* positively regulates melanin biosynthesis in mycelia of *M. graminicola* (Choi and Goodwin 2011), but negatively controls mycelial melanin production in *C. heterostrophus* (Wu et al. 2012) and *F. fujikuroi* (Wiemann et al. 2010). *VosA* in *C. sativus* negatively regulates the mycelial melanin production but positively regulate the conidial melanin production (Wang et al. 2015). In this study, we found that conidial pigmentation (most likely due to production of melanin) and the expression levels of *PKS18*, the gene involved in biosynthesis pathway of melanin in *C. sativus* (Leng et al. 2012), were decreased in the *CsLaeA*, *CsVeA*, and *CsVelB* knockout mutants, suggesting that these three genes positively control melanin production in conidia of *C. sativus*, probably by regulating the expression of genes involved in melanin biosynthesis. Our results indicate the *CsVelB* and *CsVosA* play more important roles in regulating melanin production of conidia than *CsLaeA* and *CsVeA*.

Our pathogenicity tests indicated that deletion of each of the four genes (*CsLaeA*, *CsVeA*, *CsVelB* and *CsVelC*) led to a significant reduction in virulence of the mutants on barley plants. Since the appressorium production and infection structure formation were not impacted in the mutants (Fig. 7D), the reduced virulence is probably due to the decreased expression of the two genes for NRPSs, which are involved in biosynthesis of an unknown secondary metabolite required for high virulence on barley Bowman (Condon et al. 2013;

Leng and Zhong, unpublished). Previous studies have shown that *LaeA* (Bok and Keller 2004; Perrin et al. 2007) and the *velvet* proteins promote transcription of NRPS genes. For example, expression of NRPS (*NRPS2/sidE*) for biosynthesis of a putative siderophore was greatly reduced in the $\Delta laeA$ mutant of *A. fumigatus* under inducing iron-deficient conditions (Perrin et al. 2007). In *F. oxysporum*, the *LaeA*, *VeA*, and *VelB* proteins play important roles in biosynthesis of three secondary metabolites, ferricrocin, triacetylfusarinine C and beauvericin (BEA), which are synthesized by NRPSs (Lopez-Berges et al. 2013). Our previous study also showed that *CsVosA* positively regulates the two NRPS-encoding genes involved in virulence in *C. sativus* (Wang et al. 2015). This data indicate the conserved function of *velvet*-complex proteins in regulating secondary metabolism. In this study, we had shown that $\Delta CsVeA$, $\Delta CsVelB$, and their derived double knockout mutants were more significantly reduced in virulence, and the expression of NRPS genes involved in virulence was more reduced accordingly than other mutants. This result suggests that *CsVeA* and *CsVelB* play more roles in regulating the virulence than *CsLaeA* and *CsVelC*.

In summary, the *velvet*-complex proteins can coordinately and independently regulate the fungal development and secondary metabolism of the fungal cereal pathogen *C. sativus*. The working model of *velvet*-complex proteins is illustrated in Fig. 9: *CsVeA* and *CsVelB* regulate the aerial mycelia growth; *CsVelB* and *CsVosA* regulate trehalose production (Wang et al. 2015 and Supplemental Fig. 4), conidiogenesis pattern, and spore dormancy. However, all the *velvet*-complex proteins were coordinately involved in virulence probably through

regulation of the NRPS genes, although CsVeA and CsVelB make more contributions to this process than *CsLaeA* and *CsVelC*.

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Figure captions

Fig. 1. The domain architecture of *velvet*-complex proteins in *C. sativus*. The position of the conserved methyltransferase domain for CsLaeA and the *velvet* domain for *velvet* proteins

(CsVeA, CsVelB, and CsVelC) are indicated by yellow and blue arrows, respectively. The putative nuclear localization signals (NLS) are indicated by green pentagons in CsVeA and CsVelC. The amino acid lengths are noted on the right of each protein. The percentage identities between the proteins in *C. sativus* and *A. nidulans* were indicated in parentheses.

Fig. 2. *CsVeA* and *CsVelB* influence the culture morphology and aerial mycelial growth. **A.** Cultures of wild type (WT) and knockout mutants grown on V8 PDA under constant dark and constant light conditions. The conidiation sections of $\Delta CsVeA9$ and $\Delta CsVelB3$ was indicated by red arrows. **B.** Side view of plate cultures of WT strain and knockout mutants grown under constant light conditions on V8 PDA. The long, thick white aerial mycelia (indicated by blue arrow) were observed in WT, $\Delta CsLaeA9$, and $\Delta CsVelC2$, but not in $\Delta CsVeA9$ and $\Delta CsVelB3$ and their derived double knockout mutants.

Fig. 3. Tolerance of wild type and *velvet*-complex gene knockout mutants to H₂O₂ stress. **A.** Conidia of each fungal strain were harvested from cultures grown at 25 °C for 6 days on V8 PDA plates, plated on MM plates containing H₂O₂ at different concentrations (0, 1.3 and 2.6 mM) and incubated at 25 °C for 12 hours before examination for viability under a microscope. Error bars indicate the standard deviation. **B.** Mycelia plug of each fungal strain harvested from cultures grown at 25 °C for 3 days on V8 PDA plates, were plated on MM plates containing different H₂O₂ concentrations (0, 4, 6, and 8 mM) and incubated at 25 °C for 6 days before culture radius was measured. Error bars indicate the standard deviation.

Single asterisks in A and B indicate p-value <0.05, double asterisks in A and B indicate p-value <0.001 in T-test analyses in which each strain was compared with corresponding wild type at the same H₂O₂ concentration.

Fig. 4. A. Conidial productivity of wild type and knockout mutants. The strains were grown on V8 PDA plates at 25 °C under constant dark and light conditions for 7 days, and then the conidia were harvested and quantified. Error bars indicate the standard deviation. Single asterisks indicate p-value <0.05, double asterisks indicate p-value <0.001 in T-test analyses in which each strain was compared with corresponding wild type under the same condition.

B. Conidial developmental processes in *C. sativus* strains. Conidiogenesis patterns were examined under a light microscope. The blue arrow points to a new emerging conidium on the head of the older conidium. Note that wild type produces conidia in a traditional sympodial pattern whereas the *CsVelB* mutant generates conidia through both sympodial and acropetal patterns. Bars = 20 µm.

Fig. 5. Conidial morphology of wild type and knockout mutants. The strains were grown on V8 PDA plates at 25 °C under constant dark and light conditions for 7 days, and then the conidia were harvested and photographed using Olympus BX51 microscope (Olympus, Center Valley, PA, USA) with the CCD camera digital imaging system (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) under 20X magnification. The septa are indicated by blue arrows, and the unnormalized compartments are indicated by red arrows.

Fig. 6. *Velvet*-complex genes positively regulate conidial pigmentation of *C. sativus*. **A.** The strains were grown on V8 PDA plates at 25 °C under constant dark for 7 days, and then the conidia were harvested and photographed using Olympus BX51 microscope (Olympus, Center Valley, PA, USA) with the CCD camera digital imaging system (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) under 20X magnification. The software SigmaScan Pro 5 was used to estimate the color intensity of the conidia that represent the conidial melanin content. Error bars indicate the standard deviation. Single asterisks indicate p-value <0.05 in T-test analyses in which each isolate was compared with WT. **B.** RNA expression analysis of *PKS18* in conidia. The RNA was extracted from conidia harvested from the culture grown on V8 PDA under constant dark conditions at 7 days after plating for each strain. Expression level relative to the sample of WT strain is shown. Error bars represent range of fold change calculated according to standard deviation of $\Delta\Delta Ct$. Single asterisks indicate p-value <0.05 in T-test analyses in which each isolate was compared with WT.

Fig. 7. Pathogenicity tests of wild type (WT) and gene knockout mutants on barley cv. Bowman. **A.** Disease symptoms after spray-inoculation with conidia of the wild type and gene knockout mutants. The photographs were taken at 7 days after inoculation (DAI). **B.** Disease infection responses caused by each fungal strain on Bowman. For each fungal strain, the second leaves of 9 inoculated plants were rated at 7 DAI using a 1-9 scale developed by

Fetch and Steffenson (1999). Error bars indicate the standard deviation. An asterisk represents p-value <0.05 in T-test analysis when each isolate was compared with wild type.

C. Conidial viability of WT and mutants on barley leaves. The viability was calculated as percentage of viable conidia in the total number of conidia examined at 12 hours after inoculation. Error bars indicate the standard deviation. Asterisk represents p-value <0.05 in T-test analysis when each isolate was compared with WT.

D. Appressorial formation rates of the wild type and mutants. Conidia of fungal strains were spray-inoculated on seedling leaves of barley cv. Bowman. After 12 and 24 hours after inoculation (HAI), leaf segments sampled were sampled and subjected to treatments according to the method as previously described (Wang et al. 2015), and then examined for appressorial formation under a compound microscope. The appressorium rate was calculated from the number of appressorium-forming conidia relative to the total number of conidia examined. Three replicates (barley leaves) were used for each fungal strain and 100 conidia were examined for each replicate. Error bars indicate the standard deviation.

Fig. 8. RNA expression analyses of two genes *NPS1* (protein ID 115356) and *NPS4* (protein ID 140513) involved in virulence on barley cv. Bowman. **A.** RNA expression analyses of *NPS1* (protein ID 115356) during infection of barley cv. Bowman. Expression levels relative to the sample M96 from WT is shown. Error bars represent range of fold change calculated according to standard deviation of $\Delta\Delta Ct$. The expression of the β -actin-encoding gene (*ACT*) was used as a reference. Single asterisks indicate p-value <0.05 , double asterisks indicate p-

value <0.001 in T-test analyses in which each isolate was compared with corresponding wild type at the same sample type. **B.** RNA expression analyses of *NPS4* (protein ID 140513) during infection of barley cv. Bowman. Expression levels relative to the sample M96 from WT is shown. Error bars represent range of fold change calculated according to standard deviation of $\Delta\Delta Ct$. The expression of the β -actin-encoding gene (*ACT*) was used as a reference. Single asterisks indicate p-value <0.05 , double asterisks indicate p-value <0.001 in T-test analyses in which each isolate was compared with corresponding wild type at the same sample type. All experiments were repeated at least three times.

Sample M96: RNA was extracted from a mixture of mycelia harvested from the culture at 96 HAI from different media including PDA, MM, V8 PDA, and water agar

Sample 12HAI: RNA was extracted from the inoculated barley leaves at 12 HAI on Bowman

Sample 24HAI: RNA was extracted from the inoculated barley leaves at 24 HAI on Bowman

Sample 48HAI: RNA was extracted from the inoculated barley leaves at 48 HAI on Bowman

Fig. 9. Working model of *velvet*-complex proteins in *C. sativus* that was modified from the model of LaeA and *velvet* family regulatory proteins in *A. nidulans* (Bayram et al. 2010). The solid lines indicate the major promotion or suppression effects of proteins whereas the dotted lines indicate the minor effects.

Table 1. Conidiophore productivity of wild type and knockout mutants under different light conditions

Strains	Conidiophore production (%)	
	Constant dark	Constant light

WT	100	94 ± 4.1
$\Delta CsLaeA9$	90 ± 4.7*	32 ± 3.3*
$\Delta CsVeA9$	685 ± 24.4**	57 ± 5.3*
$\Delta CsVelB3$	468 ± 20.6**	52 ± 3.7*
$\Delta CsVelC2$	96 ± 2.3	98 ± 1.1

Single conidium of wild type and mutant strains were inoculated on the centers of V8 PDA plates and allowed to grow for 7 days at 25 °C under constant dark and constant light conditions. The number of conidiophores per square centimeter of medium plates (only the conidiation sections were considered for $\Delta CsVeA9$ and $\Delta CsVelB3$) was counted with a dissecting microscope. The conidiophore production of wild type grown under constant dark condition was used as a reference (100%) to calculate the conidiophore productivity rate. Single asterisks indicate p-value <0.05, double asterisks indicate p-value <0.001 in T-test analysis when each isolate was compared with WT under constant dark condition.

Table 2. Percentage of small sized conidia of wild type and knockout mutants under different light conditions

	Dark	Light
WT	6.4 ± 1.1	8.1 ± 2.0
<i>ΔCsLaeA9</i>	53.4 ± 3.1**	6.3 ± 0.8
<i>ΔCsVeA9</i>	79.6 ± 9.2**	29.3 ± 2.9**
<i>ΔCsVelB3</i>	45.5 ± 5.4**	27.4 ± 5.3**
<i>ΔCsVelC2</i>	5.0 ± 0.8	4.4 ± 2.1
<i>ΔCsVosA5</i>	29.0 ± 4.3**	14.0 ± 2.2*
<i>ΔCsLaeAVeA2</i>	86.4 ± 6.9**	25.4 ± 4.7**
<i>ΔCsLaeAVelB3</i>	82.6 ± 8.6**	20.3 ± 3.1**
<i>ΔCsVelBVeA6</i>	92.8 ± 8.3**	28.1 ± 2.2**

Normal sized conidia were measured at 15-28 × 80-120 μm (area value 3000~4500 in SigmaScan Pro 5 software) with 4-8 septa, whereas the small sized conidia were measured at 7-14 × 15-30 μm (area value 500~1500 in SigmaScan Pro 5 software) with 0-2 septa. Single asterisks indicate p-value <0.05, double asterisks indicate p-value <0.001 in T-test analysis when each isolate was compared with WT under constant dark or constant light conditions.