The regulatory gene *VosA* affects conidiogenesis and is involved in virulence of the fungal cereal pathogen *Cochliobolus sativus*

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Abstract

VosA is one of the four components in the *velvet* complex shown to be involved in regulation of fungal development and secondary metabolism in filamentous fungi. However, the function of VosA has only been studied in a few plant pathogenic fungi. In this study, we identified the ortholog (*CsVosA*) of *VosA* in the cereal spot blotch pathogen *Cochliobolus sativus* and generated gene knockout mutants for functional characterization of the gene. Conidia of the *CsVosA* knockout mutants (*CsVosA*) lacked trehalose, were significantly reduced in viability, had less pigmentation, and showed a dramatic reduction in tolerance to heat, oxidative and ion stresses. However, $\Delta CsVosA$ produced more conidia than the wild type under both constant dark and constant light conditions, suggesting that *CsVosA* is a negative-feedback regulator in conidiation. Interestingly, the $\Delta CsVosA$ mutants exhibited a hypermorphic conidiation phenotype with indeterminate growth of the conidial tip cells resulting in head-to-tail (acropetal) arrays of conidiogenesis, indicating that some genes involved in conidiation are also regulated by *CsVosA*. The $\Delta CsVosA$ mutants showed significant reduction in virulence on susceptible barley plants and the two genes for nonribosomal peptide synthetases (NRPSs) involved in virulence during host infection were down-regulated in $\Delta CsVosA$, suggesting that *CsVosA* may affect virulence of the fungus by regulating the expression of the genes for NRPSs, as well as other genes directly or indirectly involved in virulence.

Key words: *Velvet* protein; regulator; mutant; conidiogenesis; secondary metabolites; virulence

1. Introduction

Cochliobolus sativus (Ito & Kurib.) Drechs. ex Dastur. [anamorph: *Bipolaris sorokiniana* (Sacc.) Shoem.] is a filamentous fungus belonging to the family Pleosporaceae in the order Pleosporales of Dothideomycetes. It causes several important diseases, including common root rot, spot blotch,

1 and black point in barley and wheat (Mathre 1997; Wiese 1987). Three pathotypes (0, 1 and 2) of
2 the pathogen have been identified based on virulence patterns on three barley differential lines
3 (ND 5883, Bowman and ND B112) (Valjavec-Gratian and Steffenson 1997b). Pathotype 0
4 isolates (represented by ND93-1) have low virulence on all three barley differential lines.
5 Pathotype 1 isolates (represented by ND85F) exhibit high virulence on ND 5883 but low
6 virulence on Bowman and ND B112, while pathotype 2 isolates (represented by ND90Pr) show
7 high virulence on Bowman but low virulence on ND 5883 and ND B112 (Valjavec-Gratian and
8 Steffenson 1997b). Genetic analysis and molecular mapping indicated that a single locus (*VHvI*)
9 controls the high virulence of ND90Pr on Bowman (Valjavec-Gratian and Steffenson 1997a;
10 Zhong et al. 2002). Recent comparative genome sequence analyses revealed that the *VHvI* locus
11 contains two genes (protein IDs 115356 and 140513 in [http://genome.jgi-psf.org/Cocsa1/Cocsa.
12 home.html](http://genome.jgi-psf.org/Cocsa1/Cocsa.home.html)) for nonribosomal peptide synthetases (NRPSs) unique to the *C. sativus* isolate
13 ND90Pr (Condon et al. 2013). Deletion of either of these two NRPS encoding genes led to
14 mutants with significantly reduced virulence on the barley cultivar Bowman (Condon et al. 2013;
15 Leng and Zhong, unpublished). This result demonstrated that the virulence factor in ND90Pr is a
16 secondary metabolite peptide synthesized by NRPSs. However, global regulation of the
17 secondary metabolite biosynthesis in this fungus remains to be investigated.

18
19 Fungal morphological development and secondary metabolism can be regulated by many
20 genes or gene families. The four components (VeA, VelB, VelC and VosA) of the *velvet*
21 complex have been shown to be regulators for these processes in several fungal species (Bayram
22 and Braus 2012; Bayram et al. 2008; Jiang et al. 2012; Kim et al. 2013). Among them, VeA,
23 VelB, and VelC are highly conserved in many fungal species, especially in ascomycetes (Lan et
24 al. 2014). VeA and VelB have been extensively studied in a number of fungi (Baba et al. 2012;
25 Chang et al. 2013; Choi and Goodwin 2011; Jiang et al. 2012; Kim et al. 2013; Lan et al. 2014;
26 Li et al. 2006; Lopez-Berges et al. 2013; Wiemann et al. 2010). Unlike the other three velvet
27 proteins, VosA is less conserved in ascomycetes and has not been found so far in *Fusarium*

1 pathogens (*F. graminearum*, *F. verticillioides*, *F. solani*, and *F. oxysporum*) (Lan et al. 2014;
2 Lopez-Berges et al. 2013). The gene *VosA* (for viability of spores), first identified in *Aspergillus*
3 *nidulans*, is essential for the long-term viability of spores (Bayram et al. 2010; Ni and Yu 2007)
4 and for the regulation of conidial maturation and conidiogenesis (Ahmed et al. 2013). The
5 deletion of *VosA* in *A. nidulans* results in mutant spores lacking trehalose, losing cytoplasm and
6 organelles, and dramatically reducing viability, and being more sensitive to heat and oxidative
7 stresses (Ni and Yu 2007). *VosA* also exerts negative feedback control of conidiation by down-
8 regulating *brlA* expression in *A. nidulans* (Ni and Yu 2007). In the dimorphic human pathogen
9 *Histoplasma capsulatum*, *Ryp2* (a homolog of *VosA*) is essential for the temperature-dependent
10 transition from the saprophytic filamentous phase to the single-cell pathogenic yeast form
11 (Webster and Sil 2008). Similar to the functions of the *A. nidulans VosA*, the *ryp2* mutants were
12 reduced in spore viability in *H. capsulatum* (Webster and Sil 2008). In *A. fumigatus*, *VosA* exerts
13 negative feedback control of conidiation by down-regulating *brlA* expression as found in *A.*
14 *nidulans* (Ni and Yu 2007; Park et al. 2012). In *Penicillium chrysogenum*, the *PcVosA* knockout
15 strain showed a severe conidiation defect with a reduced sporulation of about 70% compared to
16 the reference strain, indicating that *PcVosA* positively regulates conidiation (Kopke et al. 2013).
17 It was also found that viability (germination rate) of the $\Delta PcVosA$ mutant spores was
18 approximately 20% of that of the reference strain after 4 days of culture, but gradually increased
19 as the culture time lengthened (7, 10, and 13 days) and finally was close or identical to that of the
20 reference strain (Kopke et al. 2013). In *Ustilago maydis*, the knockout mutants of *umv1* (ortholog
21 of *VosA*) failed to induce galls or teliospores in maize. Chlorazol black E staining of leaves
22 infected with $\Delta umv1$ dikaryons revealed that the $\Delta umv1$ hyphae did not proliferate normally and
23 were blocked developmentally before teliospore formation (Karakkat et al. 2013). More recently,
24 Kim et al. (2014) showed that the *VosA* ortholog (*MoVOSA*) in the rice blast pathogen
25 *Magnaporthe oryzae* appeared dispensable for the fungal development and pathogenicity.
26 However, in the Southern corn leaf blight pathogen *Cochliobolus heterostrophus*, *Vos1* plays a

positive regulatory role in sexual reproduction, but negatively regulates production of asexual spores (Wang et al. 2014). It also positively regulates conidial morphology, such as the size, septum number, germination rate, as well as tolerance to oxidative and thermal stresses in *C. heterostrophus* (Wang et al. 2014). All these studies indicate that although *VosA* is conserved among filamentous fungi, its function on regulating fungal development and biosynthesis of secondary metabolites may be similar or unique depending on the fungal species involved. In this study, we explored the role of the *VosA* ortholog, *CsVosA*, in regulating morphological development, secondary metabolism and pathogenicity in *C. sativus*.

2. Materials and Methods

2.1 Fungal isolates, media and general growth conditions

The *C. sativus* isolate ND90Pr (ATCC 201652) was used as the wild type (WT) in this study. Unless mentioned otherwise, all isolates were grown on V8 potato dextrose agar (V8 PDA) under 14 hour fluorescent light at approximately 23 °C as described by Leng et al. (2011). Potato dextrose agar (PDA), minimal medium (MM) (Tinline et al. 1960), and water agar plates were also used for fungal growth in specific experiments.

2.2 Identification of the *VosA* ortholog in *C. sativus*

The *A. nidulans* *VosA* (accession number: DQ856465.1) protein sequence was 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. Pfam (<http://pfam.xfam.org/search>) was used to predict conserved domains of proteins. The nucleotide and protein sequences of *CsVosA* and *CsVosA* can be found in GenBank under the accession numbers XM_007697459.1 and EMD67942.1, respectively.

2.3 Phylogenetic analyses

The *velvet* protein sequences (VeA, VelB, VelC and VosA) in different fungi were downloaded from the GenBank database and used for phylogenetic analysis. Their accession numbers in GenBank database or the protein IDs in DOE Joint Genome Institute are listed here: AF109316_1 for AnVeA in *A. nidulans*, ABC41691.1 for AfVeA in *A. flavus*, AEY76160.1 for FgVeA in *F. graminearum*, ABC02879.1 for FvVeA in *F. verticillioides*, BAL61195.1 for PcVeA in *P. citrinum*, 1113718 for ChVeA in *C. heterostrophus*, MGG_08556 for MoVeA in *M. oryzae*, 96012 for CcVeA in *C. carbonum*, 107450 for CvVeA in *C. victoria*, 258482 for CsVeA in *C. sativus*, ABQ17967.1 for AnVelB in *A. nidulans*, ADF30048.2 for AfVelB in *A. flavus*, KDE81507.1 for AoVelB in *A. oryzae*, KDB12648.1 for VvVelB in *Villosiclava virens*, XP_001389053.2 for AnigVelB in *A. niger*, EEY18383.1 for VaVelB in *Verticillium alfalfa*, EGE01484.1 for TeVelB in *Trichophyton equinum*, EED18701.1 for TsVelB in *Talaromyces stipitatus*, XP_003714606.1 for MoVelB in *M. oryzae*, 33742 for ChVelB in *C. heterostrophus*, 38610 for CsVelB in *C. sativus*, 38540 for CvVelB in *C. victoria*, 151278 for SmVelB in *Septoria musiva*, ABQ17968.1 for AnVelC in *A. nidulans*, EAS37273.2 for CiVelC in *Coccidioides immitis*, EEQ90635.1 for AdVelC in *Ajellomyces dermatitidis*, EEH21254.1 for PbVelC in *Paracoccidioides brasiliensis*, EHA49421.1 for MoVelC in *M. oryzae*, XP_386950.1 for FgVelC in *F. graminearum*, 114528 for ChVelC in *C. heterostrophus*, 119375 for CsVelC in *C. sativus*, 31414 for CvVelC in *C. victoria*, 148540 for SmVelC in *S. musiva*, ABI51618.1 for AnVosA in *A. nidulans*, ADE45325.1 for AfVosA in *A. flavus*, XP_007818165.1 for MaVosA in *Metarhizium anisopliae*, XP_007809716.1 for MacVosA in *M. acridum*, ABQ18268.1 for AfuVosA in *A. fumigatus*, XP_003001593.1 for VaVosA in *V. alfalfa*, EHA48826.1 for MoVosA in *M. oryzae*, 1067256 for ChVosA in *C. heterostrophus*, 62627 for CsVosA in *C. sativus*, 48009 for CvVosA in *C. victoria*, 50595 for SmVosA in *S. musiva*. Alignment of the protein sequences was made with

1 ClustalX 1.8. A phylogenetic tree was constructed using the neighbor-joining method in MEGA
2 6.0 (Tamura et al. 2013). Bootstrap values were expressed as a percentage of 1000 replicates.

3 4 **2.4 Gene replacement and complementation**

5 *CsVosA* was deleted and replaced with a 2.6-kb fragment carrying the *Escherichia*
6 *coli* hygromycin phosphotransferase gene (*hph*) using the split-marker system (Catlett et al.
7 2003). The protocol previously described by Leng and Zhong (2012) was used for
8 transformation. Complementation was conducted using a co-transformation method. The plasmid
9 pII99 carrying the *NPT II* gene for G418 resistance (Beck et al. 1982) was linearized
10 with *EcoRV* and then used to co-transform with the coding and flanking sequences of *CsVosA*
11 into the *CsVosA* knockout mutant ($\Delta CsVosA5$). The primers used for gene deletion and
12 complementation are listed in Table 1. The procedure for Southern hybridization and PCR
13 confirmation was the same as previously described by Leng and Zhong (2012). Genomic DNA
14 from the wild type strain and the deletion mutants were digested by *EcoRI* and then transferred
15 to Hybond N+ (Amersham Biosciences, Piscataway, NJ, USA). The probes used to confirm the
16 deletion of the *CsVosA* gene are indicated in Fig. 2A. Sequences of primers used for probe
17 amplification and other PCR experiments are listed in Table 1.

18 19 **2.5 Spore viability test on minimal medium (MM)**

20 To test spore viability on MM, the wild type (ND90Pr), $\Delta CsVosA$ and complemented strains
21 were plated on V8 PDA. After growth at 25 °C for 6, 10, 14, 18, 29 and 33 days, conidia were
22 harvested, spread inoculated on MM, and incubated at 25 °C for 12 hours before examination for
23 viability under a microscope. The spore viability was calculated as the percentage of viable
24 conidia in the total number of conidia examined. Only those conidia producing hyphae at least 5
25 times longer than the conidia after 12 hours of incubation on MM were considered viable since
26 some of the mutant conidia premature germinated when they were attached to the conidiophores

but they failed to grow on MM. Three replicates (MM plates) were used for each fungal strain and 100 conidia were examined for each replicate.

2.6 Trehalose assay

The trehalose amounts in conidia and mycelia were measured according to previously published protocols (Ni and Yu 2007; Al-Bader et al. 2010). Briefly, conidia (10^5) and mycelia (30 mg) of each fungal strain were harvested from V8 PDA and PDB, respectively, and dried in a freeze dryer. The fungal samples were re-suspended in 200 μ l of ddH₂O and incubated in boiled water for 20 minutes. Then, the supernatants were collected by centrifugation, mixed with equal volume of 0.2 M sodium citrate (pH 5.5) and incubated at 37 °C overnight after adding 6 mU of trehalase (Sigma). The amount of glucose generated by trehalase was assayed with the glucose assay kit (Sigma) following manufacturer's instructions, and used to calculate the amount of trehalose. The sample without trehalase treatment served as a negative control. For each sample, three replicates were conducted.

2.7 Assays of thermal, oxidative, and ion stresses

To test sensitivity of spores to thermal stress, conidia of wild type, knockout mutants and complemented strain were harvested from cultures grown at 25 °C for 6 days on V8 PDA plates, plated on MM and incubated at 40 °C for 0, 1, 2, 3 hours, respectively, and then returned to 25 °C for 12, 11, 10, and 9 hours before viability of conidia was examined. Furthermore, the conidia were also incubated in a water bath at 50 °C for 0, 1.5, 3.5 minutes, respectively, and then plated on MM and incubated at 25 °C for 12 hours before viability of conidia was examined. To test the response 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. To test the response to ion (NaCl) stress, 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

different NaCl concentrations (0, 0.6 and 1.0 M) and incubated at 25 °C for 12 hours before viability of conidia was examined.

2.8 Examination of conidial size and pigmentation

To examine the size and pigmentation of conidia, the wild type and mutants were grown on V8 PDA under constant dark and constant light conditions for 7 days. Conidia of each strain were harvested and photographed under constant settings with identical white balance 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 20× 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. The average pixel brightness intensities (BI) of the conidia on the pictures were also measured using the SigmaScan Pro 5 software. The pigment intensity was converted from the pixel brightness intensity using $PI = 1 / BI$.

2.9 Analysis of conidial productivity, conidiation pattern and conidiophore production

To measure conidial productivity, a single conidium of the wild type, knockout mutants and the complemented strains was placed on the centers of separate V8 PDA plates and allowed to grow for 7 days at 25 °C under constant dark and 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 conidia formation, the conidia were inoculated on a slide culture with MM. After 5 days of growth, conidiation patterns were examined and photographed using an Olympus BX51 microscope with the CCD camera digital imaging system under the 20× magnification.

1 The conidiophore production was measured according to the methods described by
2 Chang et al. (2004). Briefly, a single conidium of the wild type and a knockout mutant was
3 placed on the centers of separate V8 PDA plates and allowed to grow for 7 days at 25 °C under
4 constant dark and constant light conditions. The number of conidiophores per square centimeter
5 of medium plates was counted with a dissecting microscope. The conidiophore production of
6 wild type grown under constant dark conditions was used as a reference to calculate the
7 conidiophore productivity rate.

9 **2.10 Examination of premature germination**

10 Conidial suspensions at 1.2×10^3 spores/mL were prepared from each culture grown on V8 PDA
11 under constant dark and constant light conditions for 3, 5, and 7 days after point inoculation with
12 hundreds of conidia, respectively. Then, 0.1 mL of conidial suspension was spread on slides and
13 the germ tube emergence was examined under a microscope. The percentage of germinated
14 spores was calculated. This test was performed three times for each strain.

16 **2.11 Pathogenicity test**

17 Pathogenicity tests were performed by spray inoculation with conidial suspension on 10~12 days
18 old seedlings of barley (cv. Bowman) plants (when the second leaves were fully expended)
19 according to Fetch and Steffenson (1999), except the concentration of the conidial suspension
20 was at 1.5×10^3 conidia/mL. Inoculated plants were incubated in a humidity chamber for 20-
21 24 hours, and then transferred into a greenhouse (23 ± 2 °C) and incubated for 6 days before the
22 spot blotch disease was rated using the 1–9 rating scale of Fetch and Steffenson (1999). For each
23 fungal strain, at least nine plants were used for inoculation and the experiment was repeated three
24 times. The photographs of infected leaves were scanned using Epson Expression 10000XL
25 (Epson America, Inc. Long Beach, CA, USA).

27 **2.12 Spore viability test on barley leaves**

To test spore viability on barley leaves, conidia (5×10^3 conidia/mL) of the wild type (ND90Pr), $\Delta CsVosA$ and complemented strains grown on V8 PDA for seven days were harvested and spray inoculated on 10~12 days old seedlings of barley cv. Bowman. The inoculated plants were misted with distilled water and then covered with a plastic bag to maintain high humidity. After 12 h of incubation under dark condition at room temperature ($23 \pm 2^\circ\text{C}$), leaf segments 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 at 12 hours post-incubation were considered viable. Three replicates (cones with three plants per cone) were used for each fungal strain and 100 conidia were examined for each replicate.

2.13 Examination of fungal infection process

To examine appressorium formation of the wild type and deletion mutants, seedlings of barley cv. Bowman were inoculated with a spore suspension (5×10^3 conidia/mL) prepared from each fungal strain. At 12 and 24 hours after inoculation (hai), leaf segments were sampled and subjected to treatments according to the method described by (Keogh et al. 1980). The stained fungal structures were examined using an Olympus BX51 microscope (Olympus, Center Valley, PA, USA) and photographed by a CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). The average rate of appressorium formation was calculated for each fungal strain based on the percentage of appressorium-forming spores relative to the total number of germinated spores examined on three inoculated leaves.

2.14 RNA extraction and gene expression analyses

For expression analyses of *CsVosA* (protein ID: 22437), *CsTpsA* (protein ID: 200256), *CsOrlA* (protein ID: 35195), *CsTpsC* (protein ID: 38836), *CsTreA* (protein ID: 39633), and *CsTreB* (protein ID: 78439), RNA was extracted from conidia harvested from the culture in V8 PDA at 7

days after inoculation (sample C), and from mycelia grown in PDB at 12 and 48 hai (sample 12M and 48M) as well as from a mixture of conidia and mycelia harvested from the culture in V8 PDA at 6 days after inoculation (sample 6DM). For expression analyses of *PKS18* (protein ID: 184740), *CsBr1A* (protein ID: 136667), *CsMedA* (protein ID: 219901), *CsStuA* (protein ID: 302496), and *CsWetA* (protein ID: 345897), total RNA was extracted from sample C, sample 12M, and sample 48M. For expression analyses of the two NRPS encoding genes (protein IDs 115356 and 140513), 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 96M) as well as from inoculated barley leaves at 12 and 24 hours hai on Bowman (sample 12B and 24B). All fungal materials were shock-frozen with liquid nitrogen immediately after harvesting. RNA was extracted using SV Total RNA Isolation System (Promega) and purified by treatment with DNase I (New England Biolabs). cDNA generation and RT-PCR procedures were the same as previously described by Leng and Zhong (2012). The β -actin-encoding gene (ACT) was amplified with the RT-Actin-F and RT-Actin-R primers (Table 1) and used as reference in all experiments. All gene expression experiments were repeated at least three times. The primer pairs for each gene used in each analysis are listed in Table 1.

3. Results

3.1 Identification of VosA homologs in *C. sativus*

Four candidate orthologs of VosA were identified from the draft genome sequence of *C. sativus* (<http://genome.jgi-psf.org/Cocsa1/Cocsa1.home.html>) through homology search using the BLASTP algorithm and VosA of *A. nidulans* as a query. The ortholog with the highest score (452) and lowest E value (8.34×10^{-44}) was identified as CsVosA (Fig. 1A), and the other three orthologs were designated as CsVeA, CsVelB and CsVelC, respectively, based on their similarities to other *velvet* proteins (VeA, VelB and VelC) in *A. nidulans*. Phylogenetic analysis indicated that the four *velvet* proteins of *C. sativus* belonged to four distinct clades, each

containing the respective *velvet* protein of different fungi (Fig. 1B). The *CsVosA* gene contains 1050 base pairs without introns and encodes a protein (349 amino acids) with a highly conserved *velvet* domain, a characteristic of *velvet* gene family, as identified using Pfam (<http://pfam.xfam.org/search>) (Fig. 1A). A putative nuclear localization signal (pat4: 277 KKRR 280 or pat7: 274 PLAKKRR 280) was also identified by using the PSORT II program (<http://psort.hgc.jp/form2.html>).

3.2 Generation of gene knockout mutants and complemented strains

Three independent deletion mutants ($\Delta CsVosA5$, $\Delta CsVosA6$ and $\Delta CsVosA7$), as well as one ectopic strain $\Delta CsVosAEC$, were generated. By introducing the *CsVosA* gene back into one of the deletion mutants, $\Delta CsVosA5$, three complemented strains ($\Delta CsVosAC1$, $\Delta CsVosAC2$, and $\Delta CsVosAC3$) were obtained. These knockout mutants and complemented strains were confirmed by PCR amplification and Southern hybridization analyses. As shown in Fig. 2A and B, an amplicon (235 bp) was only present in the wild type (ND90Pr) and complemented strains but not in the knockout mutants, when the *CsVosA*-specific primer pair SP-*CsVosA*-F and SP-*CsVosA*-R were used for PCR amplification. Using the primer *CsVosA*-F0 at the flanking region of *CsVosA* and the primer HYG2-R at the hygromycin resistance gene (*hph*) for PCR, the fragment (2,830bp) was only amplified from the knockout mutants but not from the wild type, indicating that *CsVosA* was replaced by *hph*. The deletion of *CsVosA* in $\Delta CsVosA5$ was further confirmed by Southern hybridization of the *Eco*RI-digested genomic DNA from the wild type and mutants using a probe amplified from the gene encoding *CsVosA* using primer pair SP-*CsVosA*-F and SP-*CsVosA*-R (Table 1). As indicated in Fig. 2C, A 9.6 kb fragment in the wild type strain (ND90Pr) and a 7.5 kb fragment in the complemented strain ($\Delta CsVosAC1$) were detected while no hybridization signal was observed in the $\Delta CsVosA$ strain ($\Delta CsVosA5$).

3.3 mRNA expression of *CsVosA* at different growth stages

As expected, no mRNA expression of *CsVosA* was observed in the deletion mutant ($\Delta CsVosA5$) at the different growth stages (Fig. 3). However, mRNA expression of *CsVosA* was significantly higher in conidia than in vegetative mycelia in the wild type and the complemented strain (Fig. 3). Also, *CsVosA* showed a higher mRNA expression in the complemented strain than in the wild type at the three different growth stages. The higher expression of *CsVosA* in the complemented strain may be due to the gene inserted into a highly expressed region although further research is needed.

3.4 *CsVosA* regulates spore viability, trehalose accumulation, and tolerance to thermal and oxidative as well as ion stresses

As shown in Fig. 4A, the survival rates of conidia from $\Delta CsVosA$ grown for 10 or more days on MM were significantly reduced, while those of conidia from wild type and the complemented strain had little changes during 33 days of the incubation. Close microscopic examination further indicated that many of the mutant conidia lost cytoplasm and totally collapsed (data not shown).

The amount of trehalose in the $\Delta CsVosA$ mutant was significantly reduced in conidia but significantly increased in mycelia compared to those from the wild type and the complemented strain (Fig. 4B and 4C). To determine the role of *CsVosA* in regulating genes involved in synthesis and breakdown of trehalose, the expression levels of three genes (*CsTpsA*, *CsOrlA*, *CsTpsC* orthologous to *A. nidulans tpsA*, *orlA*, *tpsC*, which are involved in trehalose synthesis) and two genes (*CsTreA* and *CsTreB* orthologous to *A. nidulans treA* and *treB*, which are involved in trehalose breakdown) (Al-Bader et al. 2010; Ni and Yu 2007), were measured by quantitative real time PCR. The results showed that mRNA levels of *CsTpsA* and *CsOrlA* were significantly reduced in the $\Delta CsVosA$ mutant during asexual development (formation of conidia) compared to the wild type and complemented strain (Fig. 4D). However, mRNA levels of *CsTreA* (but not *CsTreB*) were significantly increased in mature conidia.

1 The $\Delta CsVosA$ mutant conidia exhibited a drastic reduction in tolerance to heat (Fig. 5A
2 and B), H_2O_2 (Fig. 5C), and ion (NaCl) (Fig. 5D) stresses than present in conidia of the wild type
3 and complemented strains. Reintroduction of the *CsVosA* gene into the mutants restored the wild
4 type tolerance (Fig. 5).

6 **3.5 *CsVosA* regulates asexual spore production and conidiogenesis pattern**

7 The $\Delta CsVosA$ mutant strain produced 2.25 and 1.66 folds more conidia than the wild type on V8
8 PDA under both constant dark and light conditions, respectively (Fig. 6), indicating that *CsVosA*
9 represses spore production in *C. sativus*. In addition, the mutant produced more conidia under
10 constant dark conditions than under constant light conditions, whereas the wild type produced a
11 similar number of conidia under all these growth conditions (Fig. 6).

12 Further examination of conidiogenesis indicated that no significant differences in
13 conidiophore production were observed between wild type and the $\Delta CsVosA$ mutant under the
14 constant light conditions, but the mutant generated more conidiophores under constant dark
15 conditions compared to the wild type (Table 2). More interestingly, the conidiation pattern was
16 different between wild type and the knockout mutant. The wild type produced 3-5 conidia on a
17 conidiophore in a sympodial fashion, while the $\Delta CsVosA$ mutant had conidiophores with multiple
18 chains of conidia with the oldest conidium at the base and the youngest one emerging at the tip
19 of the spore chain (Fig. 7). The wild type produced conidia in a sympodial pattern, one fashion of
20 the holoblastic differentiation, while the $\Delta CsVosA$ mutant produced spores by two patterns of
21 holoblastic differentiation: sympodial and acropetal.

22 To further investigate the molecular regulatory role of *CsVosA* in conidiation, we
23 examined mRNA levels of four *C. sativus* genes [(*CsBrIA* (protein ID: 136667), *CsWetA* (protein
24 ID:345897), *CsStuA* (protein ID: 302496) and *CsMedA* (protein ID: 219901)] orthologous to
25 *brIA* (accession number M20631.1), *wetA* (accession number M60528.1), *stuA* (accession

number M83569.2), and *medA* (accession number AF080599.1), which are involved in the regulatory pathway of sporulation and spore maturation in *A. nidulans* (Adams et al. 1988; Marshall and Timberlake 1991; Miller et al. 1992). As shown in Fig. 8, *CsBrlA*, *CsStuA* and *CsWetA* were down-regulated while *CsMedA* was up-regulated in $\Delta CsVosA5$ conidia. However, a very low level of *CsBrlA* expression was detected in mycelia grown in PDB for 12 and 48 hours, indicating that this gene is preferably expressed in conidia. The expression levels of *CsWetA*, *CsStuA* and *CsMedA* were very low in mycelia after 12 hours of growth, but increased after 48 hours of growth. Also, the expression of *CsWetA*, *CsStuA* and *CsMedA* was up-regulated in $\Delta CsVosA5$ mycelia after 48 hours of growth in PDB as compared to the wild type and the complemented strain.

3.6 *CsVosA* regulates conidial size

$\Delta CsVosA$ conidia differed from WT conidia in size, shape, and septa production (Fig. 9). The wild type strain (ND90Pr) produced normal sized conidia measured at $15\text{-}28 \times 80\text{-}120 \mu\text{m}$ (area value 6000~12000 in SigmaScan software) with 4-8 septa, and 4% or 12% small conidia measured at $7\text{-}14 \times 15\text{-}30 \mu\text{m}$ (area value 2000~3000 in SigmaScan software) with 0-1 septa under constant dark and constant light conditions, respectively (Table 3). However, the $\Delta CsVosA$ mutant produced more small conidia and many large conidia measured at $20\text{-}35 \times 120\text{-}250 \mu\text{m}$ (area value 12000~28000 in SigmaScan software) under both constant dark and continuous light conditions, respectively, compared to the wild type (Table 3). For these large conidia, the number of septa/conidium ranged from 0-8. However, no significant differences were found in germination rate and responses to the stresses tested among the different sized conidia from each of the strains (data not shown).

3.7 *CsVosA* is required for spore dormancy

1 The wild type conidia of *C. sativus* would not germinate when they were attached to the
2 conidiophores. However, the $\Delta CsVosA$ mutant conidia germinated when they were still attached
3 to the conidiophores (Fig. 10). Premature germination rate was even higher for the *CsVosA*
4 knockout mutants grown under light conditions than under dark conditions (Fig. 10). This
5 indicates that *CsVosA* regulates spore dormancy. However, viability of the premature-
6 germinated conidia was the same as that of the conidia that germinated after detachment from the
7 conidiophore (data not shown), indicating the premature germination does not impair the
8 conidial viability.

10 **3.8 *CsVosA* regulates pigmentation**

11 The $\Delta CsVosA$ mutant conidia were lighter than the wild type conidia. However, mycelia of the
12 mutant were darker than those of the wild type after 24 and 48 hours of growth in PDB (Fig. 11A
13 and B). To investigate if *CsVosA* affects the expression of *PKS18*, the gene involved in
14 biosynthesis of melanin in *C. sativus* (Leng et al. 2011), mRNA levels of *PKS18* in the wild type,
15 $\Delta CsVosA$ mutant and complemented strains were examined using quantitative real time PCR.
16 The results showed that *PKS18* was down-regulated in $\Delta CsVosA$ mutant conidia but significantly
17 up-regulated in mycelia after 48 hours of growth in PDB (Fig. 11C).

19 **3.9 Roles of *CsVosA* in pathogenicity and virulence**

20 Both the wild type and $\Delta CsVosA$ strains produced spot blotch symptoms on Bowman (Fig. 12A).
21 However, the lesion number and size caused by the $\Delta CsVosA$ mutant were significantly different
22 from those induced by the wild type (Fig. 12B and C). The number of lesions produced by the
23 mutant per leaf was significantly lower compared to WT under the same inoculation conditions
24 (Fig. 12B). The averaged lesion size of the mutant was 68% of that of wild type (Fig. 12C). Based
25 on the 1–9 disease rating scale of (Fetch and Steffenson 1999), the infection responses caused by

the wild type and mutant strains were rated as 7-8 and 5-6, respectively, at 6 days after inoculation (dai). No significant differences in pathogenicity and virulence were observed between the wild type and the complemented strain (Fig. 12B and C).

To determine which stage of the disease development is affected by *CsVosA*, the survival rate and appressorium formation of wild type and mutant conidia on inoculated leaves of Bowman were examined under a microscope. The results showed that survival rate of the mutant conidia was significantly lower than that of wild type conidia while no significant differences were observed between the wild type and the complemented strain in this regard (Fig. 12D). However, conidia from both the wild type and mutants formed normal appressoria at 12 and 24 hai on the barley leaves, and no significant differences were observed between the $\Delta CsVosA$ and the wild type in appressorium formation frequencies for the germinated conidia (Fig. 12E).

mRNA expression levels of the two encoding genes for NRPSs (protein IDs 115356 and 140513) required for high virulence of isolate ND90Pr on Bowman (Condon et al. 2013) were measured in the wild type, $\Delta CsVosA$ and the complemented strain at different time points during host infection. The results showed that the two genes had significantly lower mRNA expression levels in the $\Delta CsVosA$ mutant compared to the wild type and complemented strain, especially at 12 (for ID 115356) and 24 hai (for IDs 115356 and 140513) (Fig. 12F and G).

4. Discussion

Although the *velvet* proteins are found to be conserved across many filamentous fungi, their functions have been characterized in some species, especially for VosA and VelC, which were only studied in a few cases (Kim et al. 2014; Wang et al. 2014). In the present study, we identified and functionally characterized the ortholog of VosA (*CsVosA*) in *C. sativus*. Our

1 results revealed that CsVosA plays similar and unique roles in regulating fungal development,
2 secondary metabolism and pathogenicity.

3 Deletion of *CsVosA* led to increased production of conidia under both constant dark and
4 light conditions, suggesting that CsVosA negatively regulates conidial productivity, just as found
5 in *A. nidulans* and *A. fumigatus* (Ni and Yu 2007; Park et al. 2012). The same effect was found
6 for *VOS1* in *C. heterostrophus*, but the *VOS1* mutant had an increased conidial production only
7 under constant dark conditions (Wang et al. 2014). These results indicate that the function of
8 VosA/Vos1 can be different under different conditions even in certain related fungal species, like
9 *C. sativus* and *C. heterostrophus*. Lights play different roles in conidiation of *C. sativus* and *C.*
10 *heterostrophus*. The wild type strain of *C. heterostrophus* produced more conidia under constant
11 light conditions than under constant dark conditions (Wang et al. 2014), whereas no significant
12 difference in conidial productivity was observed in *C. sativus* grown under these two conditions.
13 In addition, the *C. sativus* *CsVosA* mutant produced more conidia under constant dark conditions
14 than under constant light conditions (Fig. 6), indicating that *CsVosA* may have a stronger
15 suppression of conidiation in *C. sativus* under constant dark conditions than under constant light
16 conditions.

17 We observed that the *CsVosA* mutant produced many larger conidia, whereas wild type
18 conidia were uniform in size with regularly spaced septa. Wang et al. (2014) also showed that
19 deletion of *VEL2* or *VOS1* affected conidium size and spacing of septa in *C. heterostrophus*.
20 These results indicate VosA is a positive regulator of these processes. In *Fusarium verticillioides*,
21 the cell wall defected mutants produced larger conidia due to the reduced resistance of cell wall
22 to turgor pressure (Li et al. 2006). Osmotic stabilizers are able to reduce cellular turgor pressure
23 and have been shown to restore cell wall defective mutants of *S. cerevisiae* (Levin and
24 Bartletttheubusch 1992; Torres et al. 1991) and *A. nidulans* (Borgia et al. 1996) to the wild type
25 phenotypes. We examined conidial morphology of the wild type and $\Delta CsVosA$ grown on V8

1 PDA media containing the osmotic stabilizers (1.0 M sorbitol or 0.8 M sucrose), and failed to
2 detect differences in conidial size between the mutant and the wild type (data not shown),
3 indicating that *CsVosA* may regulate genes associated with biosynthesis of conidial cell wall
4 components.

5 Our results showed that deletion of *CsVosA* results in a decreased amount of trehalose in
6 conidia, and one of the genes involved in trehalose breakdown was up-regulated while two of the
7 genes involved in trehalose biosynthesis were down-regulated in the *CsVosA* mutant, indicating
8 that the corresponding protein is a positive regulator of trehalose biosynthesis. These findings are
9 consistent with those found for *A. nidulans*, *A. fumigatus* and *C. heterostrophus*, where *VosA*
10 positively regulates conidial maturation and trehalose biosynthesis (Bayram et al. 2010; Park et
11 al. 2012; Wang et al. 2014). However, the regulatory role of *VosA* in mycelial trehalose
12 metabolism of *C. sativus* appeared to be different because deletion of *CsVosA* caused trehalose
13 increase in mycelia compared to the wild type (Fig.4C).

14 We observed that $\Delta CsVosA$ conidia were hypersensitive to oxidative stress mediated by
15 H_2O_2 . This result matches those found for *VosA* in *C. heterostrophus* (Wang et al. 2014). In *C.*
16 *heterostrophus*, VOS1 (orthologous to *VosA*) was found to regulate the expression of CAT3, one
17 of three catalases in the genome central to decomposition of H_2O_2 , and therefore it provides the
18 protection from oxidative damage by ROS. *CsVosA* may have similar functions in controlling
19 the expression of CAT3, but further studies are required to reveal the role of *CsVosA* in
20 regulating CAT3 in *C. sativus*.

21 In *Cochliobolus* species, conidiogenesis follows a sympodial fashion in which conidia are
22 normally produced by an expansion and swelling of the apex of the conidiophore, and after the
23 formation of the first conidium, the active apical tip moves to the side to produce the next
24 conidium. Multiple rounds of conidiation result in three to five spores borne sympodially on a
25 conidiophore. However, in the *CsVosA* knockout mutant, we observed both sympodial and
26 acropetal conidiation patterns. Sympodial conidiation generated initial conidia from the

1 conidiophore, while acropetal conidiation produced conidial chains with the youngest conidia
2 emerging at the tip of the initial conidia. This result suggests that CsVosA regulates
3 conidiogenesis.

4 Among the four *C. sativus* genes (*CsBrlA*, *CsWetA*, *CsStuA* and *CsMedA*), only *CsMedA*
5 showed up-regulated expression while the others were down-regulated during conidiation of the
6 $\Delta CsVosA$ mutant (Fig.8). This result suggests that CsVosA negatively regulates *CsMedA* but
7 positively controls the other three genes. A mutation at *MedA* in *A. nidulans* results in aberrant
8 conidiophores with branching chains of metulae, delayed conidial differentiation, and frequent
9 re-initiation of the secondary conidiophore (Busby et al. 1996; Clutterbuck 1969; Gemes and
10 Clutterbuck 1994). The *REN1* (ortholog of *AnMedA*) mutants of *Fusarium oxysporum* lack
11 normal conidiophores and phialides, producing rod-shaped cells in a single chain at the early
12 stage of development and frequently form the cells in branching chains at the later stage of
13 development, while the wild type strain never forms conidia in a chain (Ohara et al. 2004). In
14 *Magnaporthe grisea*, deletion of *ACR1* (ortholog of *AnMedA*) caused holoblastic acropetal
15 pattern sporulation whereas the spore formation and differentiation in wild type occur in a typical
16 holoblastic sympodial fashion (Lau and Hamer 1998). We hypothesize that CsVosA affects
17 expression of *CsMedA* and others, leading to the occurrence of acropetal conidiation. More
18 functional studies of this gene and other genes related to conidiation are needed to fully
19 understand conidial development in *C. sativus*.

20 In our study, we found that pigmentation (most likely due to production of melanin) and
21 the expression levels of *PKS18*, the gene involved in biosynthesis of melanin in *C. sativus* (Leng
22 et al. 2011), were decreased in conidia but increased in mycelia of the $\Delta CsVosA$ mutant. This
23 result suggests that VosA positively and negatively affects the melanin production in conidia and
24 mycelia of *C. sativus*, respectively, by controlling the expression of genes involved in melanin
25 biosynthesis. Previous studies showed that other *velvet* proteins regulate mycelial melanin
26 production in fungi. For example, VeA positively regulates melanin biosynthesis in mycelia of

1 *Mycosphaerella graminicola* (Choi and Goodwin 2011), but negatively controls mycelial
2 melanin production in *Cochliobolus heterostrophus* (Wu et al. 2012) and *Fusarium fujikuroi*
3 (Wiemann et al. 2010). To our knowledge, the regulatory role of VosA in melanin production
4 has been shown for the first time in the present study.

5 Deletion of *CsVosA* led to a significant reduction in lesion number and size of the spot
6 blotch disease caused by the mutant on barley plants. The fewer lesion number is probably due to
7 the lower viability of the mutant conidia compared to the wild type conidia. The reduced
8 virulence is probably due to the positive regulatory role of *CsVosA* in expression of the two
9 genes for NRPSs involved in biosynthesis of an unknown secondary metabolite which is
10 required for high virulence on barley Bowman (Condon et al. 2013; Leng and Zhong,
11 unpublished). Previous studies have shown that two of the *velvet* proteins, VeA and VelB, play
12 important roles in regulating the production of secondary metabolites, including melanin in
13 *Botrytis cinerea* (Yang et al. 2013) and *C. heterostrophus* (Wu et al. 2012), fumonisin and
14 fusarin in *F. verticillioides* (Myung et al. 2009), sterigmatocystin in *A. nidulans*, trichothecene
15 and zearalenone in *Fusarium graminearum*, and penicillin in *Penicillium chrysogenum* (Hoff et
16 al. 2010; Kato et al. 2003). Wu et al. (2012) demonstrated that Vel1, the VeA ortholog in *C.*
17 *heterostrophus*, is involved in regulation of biosynthesis of T-toxin, the host selective toxin
18 (HST)/effector for pathogenicity of the pathogen. To our knowledge, our study is the first to
19 demonstrate that VosA affects expression of the genes for NRPSs involved in virulence in
20 ascomycete fungi.

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42 Figure captions

43 **Fig. 1.** The structure and phylogenetic tree of *C. sativus* *VosA* gene and its orthologs in other
44 fungal species. (A) Structural feature of *VosA* gene in *C. sativus*. The position of the conserved

1 *velvet* factor domain and a putative nuclear localization signal (NLS) are indicated by a blue
2 pentagon and a red rectangle, respectively. The nucleotide and amino acid length are noted in
3 parentheses below the gene; the percent identity and similarity between *CsVosA* and its ortholog
4 in *A. nidulans* are indicated on the right in parentheses. (B) Phylogenetic tree for *CsVosA*. The
5 phylogenetic tree was constructed by neighbor-joining method with 1000 bootstrap replicates;
6 numbers on the branches represent the percentage of replicates supporting each branch. The
7 *velvet* proteins of *C. sativus* are shaded. The scale bar represents 20% sequence divergence. The
8 accession numbers in GenBank database or the protein IDs in DOE Joint Genome Institute of the
9 genes are listed in Materials and Methods.

11 **Fig. 2.** Generation as well as PCR and southern blot confirmation of *CsVosA* and complemented
12 strains in *Cochliobolus sativus*. (A) Schematic representation of the replacement of
13 the *CsVosA* gene by a 2.6-kb fragment carrying the *Escherichia coli* hygromycin
14 phosphotransferase gene (*hph*) using the split-marker system. (B) Polymerase chain reaction
15 (PCR) amplification analysis of the wild type (ND90Pr), $\Delta CsVosA$ mutants ($\Delta CsVosA5$,
16 $\Delta CsVosA6$, $\Delta CsVosA7$) and complemented strains ($\Delta CsVosAC1$, $\Delta CsVosAC2$, $\Delta CsVosAC3$) using
17 primer pairs (SP-*CsVosA*-F/SP-*CsVosA*-R) and (*CsVosA*-F0/HYG2-R). (C) Southern
18 hybridization of *Eco*RI-digested genomic DNA from the wild type (ND90Pr), $\Delta CsVosA$ mutant
19 ($\Delta CsVosA5$), and complemented strain ($\Delta CsVosAC1$) using a probe amplified with primers
20 *CsVosA*-F1 and *CsVosA*-F2-1 and labeled by ^{32}P . The 7.6-kb fragment in the wild type strain
21 (ND90Pr) was replaced by the 5.3-kb fragment in the $\Delta CsVosA5$ strain.

23 **Fig. 3.** Expression analyses of *CsVosA* in wild type and mutants at different developmental
24 stages. Expression level relative to the sample 12M of the wild type strain (ND90Pr) 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. 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.

Sample 6DM: RNA was extracted from a mixture of conidia and mycelia harvested from the culture in V8 PDA at 6 dai

Sample C: RNA was extracted from conidia harvested from the culture in V8 PDA at the 7 dai

Sample 12M: RNA was extracted from mycelia grown in PDB at 12 hai

Sample 48M: RNA was extracted from mycelia grown in PDB at 48 hai

Fig. 4. Viability and trehalose content of conidia in wild type and mutant isolates. (A) Viability of conidia of the wild type (ND90Pr), $\Delta CsVosA$ and the complemented strain grown on V8 PDA at 25 °C for 6, 10, 14, 18, 29 and 33 days. The conidia were considered viable when they were able to germinate to produce germ tubes at least 5 times longer than the conidia after 12 hours of incubation on MM. The survival rate of conidia was calculated as the percentage of viable conidia in the total number of conidia examined. 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 at the same culture time. (B) Trehalose content in conidia of wild type, $\Delta CsVosA$ and complemented strains. Trehalose was extracted from 10^5 conidia harvested from fungal cultures grown on V8 PDA for 6 days. The amount of glucose released from trehalose by trehalase was assayed with a glucose assay kit (Sigma) and used to calculate the amount of trehalose in conidia. Each sample not treated with trehalase served as a negative control. Three replicates were used for each fungal strain. Error bars indicate the standard deviation. Asterisks represent p-value <0.05 in T-test

analyses when each isolate was compared with the wild type. (C) Trehalose content in mycelia of wild type, $\Delta CsVosA$ and complemented strains. Trehalose was extracted from 30mg mycelia harvested from fungal cultures grown in PDB for 24 and 48 hours. The amount of glucose released from trehalose by trehalase was assayed with a glucose assay kit (Sigma) and used to calculate the amount of trehalose in mycelia. Each sample not treated with trehalase served as a negative control. Three replicates were used for each fungal strain. Error bars indicate the standard deviation. Asterisks represent p-value <0.05 in T-test analyses when each isolate was compared with the wild type. (D) RNA expression of trehalose-related genes at different developmental stages. Expression level relative to the sample 12M of the wild type strain 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 strain was compared with corresponding wild type at the same sample type.

Sample 6DM: RNA was extracted from a mixture of conidia and mycelia harvested from the culture in V8 PDA at 6 dai

Sample C: RNA was extracted from conidia harvested from the culture in V8 PDA at the 7 dai

Sample 12M: RNA was extracted from mycelia grown in PDB at 12 hai

Sample 48M: RNA was extracted from mycelia grown in PDB at 48 hai

Fig. 5. Tolerance of the wild type, $\Delta CsVosA$ and complemented strain to high temperature 40 °C (A), 50 °C (B), H₂O₂ stress (C), and NaCl stress (D). (A) Conidia of each fungal strain harvested from cultures grown at 25 °C for 6 days on V8 PDA plates, were plated on MM and incubated at 40 °C for 0, 1, 2, 3 hours, respectively, and then returned to 25 °C for 12, 11, 10, and 9 hours before viability of conidia was examined. Single asterisks in A-D indicate p-value <0.05, double

1 asterisks in A-D indicate p-value <0.001 in T-test analyses in which each strain was compared
2 with corresponding wild type at the same incubation time. (B) Conidia of each fungal strain
3 harvested from cultures grown at 25 °C for 6 days on V8 PDA plates, were incubated in water
4 bath at 50 °C for 0, 1.5, 3.5 minutes, respectively, and then plated on MM and incubated at 25 °C
5 for 12 hours before viability of conidia was examined. Single asterisks in A-D indicate p-value
6 <0.05, double asterisks in A-D indicate p-value <0.001 in T-test analyses in which each strain
7 was compared with corresponding wild type at the same incubation time. (C) Conidia of each
8 fungal strain harvested from cultures grown at 25 °C for 6 days on V8 PDA plates, were plated
9 on MM plates containing different H₂O₂ concentrations (0, 1.3 and 2.6 mM) and incubated at 25
10 °C for 12 hours before viability of conidia was examined. Single asterisks in A-D indicate p-
11 value <0.05, double asterisks in A-D indicate p-value <0.001 in T-test analyses in which each
12 strain was compared with corresponding wild type at the same H₂O₂ concentration. (D) Conidia
13 of each fungal strain harvested from cultures grown at 25 °C for 6 days on V8 PDA plates, were
14 plated on MM plates containing different NaCl concentrations (0, 0.6 and 1.0 M) and incubated
15 at 25 °C for 12 hours before viability of conidia was examined. The conidia were considered
16 viable when they were able to germinate to produce germ tubes at least 5 times length of the
17 conidia. The survival rate was calculated as percentage of viable conidia in the total number of
18 conidia examined. Error bars indicate the standard deviation. Single asterisks in A-D indicate p-
19 value <0.05, double asterisks in A-D indicate p-value <0.001 in T-test analyses in which each
20 strain was compared with corresponding wild type at the same NaCl concentration.

21
22 **Fig. 6.** Conidial productivity of wild type, knockout mutant and the complemented mutant. The
23 strains were grown on V8 PDA plates at 25 °C under constant dark and light conditions for 7
24 days, and then the conidia were harvested and quantified. Error bars indicate the standard
25 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.

Fig. 7. Conidial developmental processes in *C. sativus* strains. Conidiogenesis patterns were examined under a light microscope. The white arrow points to a growing point of a new conidium and the dark arrow points to a younger conidium on the head of the older conidium. Note that wild type produces conidia in a traditional sympodial pattern whereas the knockout mutant generates conidia through both sympodial and acropetal patterns. bars = 20 μ m.

Fig. 8. RNA expression analyses of *CsBrlA*, *CsStuA*, *CsWetA* and *CsMedA* in the wild type and mutants at different developmental stages. Expression level relative to the sample 12M of the wild type strain (ND90Pr) 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.

Sample C: RNA was extracted from conidia harvested from the culture in V8 PDA at the 7 dai

Sample 12M: RNA was extracted from mycelia grown in PDB at 12 hai

Sample 48M: RNA was extracted from mycelia grown in PDB at 48 hai

Fig.9. Conidial morphology. 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. Note the wild type (ND90Pr) and complemented strains have most of conidia with uniform size and regularly spaced septa. Arrows point to mutant conidia of irregular length and to compartments without septa or with irregularly placed septa.

Fig. 10. Premature germination rate of mutant on V8 PDA under constant dark and light conditions at different time points. The germination rate was calculated as percentage of germinated conidia in the total number of conidia examined. Error bars indicate the standard deviation. Asterisks represent p-value <0.001 in T-test analyses when each isolate was compared with corresponding wild type under the same condition.

Fig. 11. *CsVosA* positively regulates conidial pigmentation of *C. sativus* but negatively regulate mycelial pigmentation of *C. sativus*. (A) 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 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, double asterisks indicate p-value <0.001 in T-test analyses in which each isolate was compared with corresponding wild type under the same culture condition. (B) Mycelial pellet were collected from cultures in PDB at 24 hours and 48 hours after inoculation with conidia and then photographed using a digital camera (Nikon D80 with lens Af-S Micro NIKKOR 60 mm). (C) RNA expression analysis of *PKS18* at different developmental stages. Expression level relative to the sample 12M of the wild type 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, 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.

Sample C: RNA was extracted from conidia harvested from the culture in V8 PDA at the 7 dai

Sample 12M: RNA was extracted from mycelia grown in PDB at 12 hai

Sample 48M: RNA was extracted from mycelia grown in PDB at 48 hai

Fig. 12. Pathogenicity and virulence of the wild type (ND90Pr) and mutants on barley cv.

Bowman. (A) Disease symptoms after spray-inoculation with conidia of the wild type, knockout mutant ($\Delta CsVosA$) and complemented strain ($\Delta CsVosAC$). The photographs were taken at 6 dai.

(B) Quantification of lesion number. For each fungal strain, a total of 25 inoculated leaves were examined for lesion numbers at 6 dai and the average lesion number per leaf was calculated.

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) Quantification of lesion size on leaves used

in (B). The average lesion size (length) caused by the wild type strain (ND90Pr) was used as a

control and relative lesion size was calculated for the knockout mutant ($\Delta CsVosA$) and

complemented strain ($\Delta CsVosAC$). Error bars indicate standard deviation. An asterisk represents

p-value <0.05 in T-test analysis when each isolate was compared with wild type. (D) Viability of

conidia on barley leaves. The survival rate was calculated as percentage of viable conidia in the

total number of conidia examined. Error bars indicate the standard deviation. An asterisk

represents p-value <0.05 in T-test analysis when each isolate was compared with the wild type

(ND90Pr). (E) Appressorial formation rate. The wild type and $\Delta CsVosA$ conidia were spray-

inoculated on seedling leaves of barley cv. Bowman. After 12 and 24 hours after inoculation, the

appressorium rate was calculated from the number of appressorium-forming conidia relative to

the total number of conidia examined. Error bars indicate the standard deviation. (F) RNA expression analyses of *NPS1* (ID 115356) during infection of barley cv. Bowman. Expression levels relative to the sample 96M 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. Asterisks indicate p-value <0.05 in T-test analyses in which each isolate was compared with corresponding wild type at the same sample type. (G) RNA expression analyses of *NPS4* (ID 140513) during infection of barley cv. Bowman. Expression levels relative to the sample 96M are 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. Asterisks indicate p-value <0.05 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 96M: RNA was extracted from a mixture of mycelia harvested from the culture at 96 hours after inoculation from different media including PDA, MM, V8 PDA, and water agar

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

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

1
2 **Table 1.** Primers used in this study

Primer	Sequence 5' to 3' *	Purpose
M13F	GACGTTGTAAAACGACGGCCAGTG	Generation of knockout mutant
M13R	CACAGGAAACAGCTATGACCATGA	
HY	GGATGCCTCCGCTCGAAGTA	
YG	CGTTGCAAGACCTGCCTGAA	
CsVosA-F1	TATAGGTCGCCATTGCCTTC	
CsVosA-F2-1	<i>CACTGGCCGTCGTTTTACAACGTCGACGCTGACGAAGAGGTAGG</i>	
CsVosA-F3	<i>TCATGGTCATAGCTGTTTCCTGTGGCCACGTCTTATCCGACAAA</i>	
CsVosA-F4	CCTCGCTGCAATAACTGAAA	
SP-CsVosA-F	ACGACAAGGACGAACCAATC	PCR confirmation
SP-CsVosA-R	TGGTAGCGCTCGAGATCTTT	
CsVosA-F0	CGGCACTTTACCTCTCTTCG	
HYG2-R	ATGTTGGCGACCTCGTATT	
SP-CsVosA-F	ACGACAAGGACGAACCAATC	Southern confirmation
SP-CsVosA-R	TGGTAGCGCTCGAGATCTTT	
CP-CsVosA-F-2	CGGCACTTTACCTCTCTTCG	Generation of complimentary mutant
CP-CsVosA-R	GTGTGCTCCGTTTTCTCAT	
Cs-brlA-L	TCCCGAAGCTATGGTGGATC	Expression of conidiation-related genes
Cs-brlA-R	CTGGCTGGAGGTTGATGGTA	
Cs-wetA-L	TACGCCCCTTCAACAACTCT	
Cs-wetA-R	GAAGCTCTGGTCACACTCCT	
Cs-medA-L	TGGTGGAAGAAAAGGACGA	
Cs-medA-R	TCGCTGTCTTGCTTTGCTTT	
Cs-stuA-L	ACCCAAAGACAGAGATGGCA	
Cs-stuA-R	ACTCATTGCCGTGATCATGC	
TpsA-F-2	TCATGCACAAGTCCGTCTCT	Expression of trehalose-related genes
TpsA-R-2	AATGAGACTGCCGTTCAAGC	
TpsB-F-1	ACTACCTTGAGCGTGTCTGAA	
TpsB-R-1	TGGCTCAATGACGACTGAGT	
CsOrlA-F-1	ATGAGCGCCCCTACAGATAC	
CsOrlA-R-1	TCGTCAAAGTAGGCGGTCTT	
Cs-treA-L	TTCGTCTCAAGCCTGGATGT	
Cs-treA-R	AGCCTGACTACTGCCTTGTT	
TreB-F-1	TCTCCAACTTGCTCCAGGAG	
TreB-R-1	GCAATGTTGGAGCCATCGAT	

CsNPS1-F1	GTCGACTGCCATCTGGAAAC	Expression of NRPS-related genes
CAN1-F2-2	TGCGGGGTCATCTTAAAAAC	
CsNPS4-RT-F3	ACAGGATCATTGGCCAGTTC	
CsNPS4-RT-R3	AAGGAGGACTTTGGCCATTT	
08856-F	GTGGTGTCATGGCATAACGAG	Expression of PKS-related gene
PKS18F4-KO	AGTGAGGAAGGAGCCATGAA	
RT-Actin-F	GTATGGGCCAAAAGGACTCA	Control for gene expression
RT-Actin-R	CACGCAGCTCGTTGTAGAAG	

1 * Italic sequences are complementary to M13F and M13R sequences, respectively.

2

Table 2. Conidiophore productivity of wild type (ND90Pr) and knockout mutant ($\Delta CsVosA5$) under different conditions

Strains	Condition	Conidiophore production (%)
ND90Pr	Constant dark	100
ND90Pr	Constant light	94 $\pm$ 4.1
$\Delta CsVosA5$	Constant dark	153 $\pm$ 7.9*
$\Delta CsVosA5$	Constant light	98 $\pm$ 2.3

Single conidium of wild type and $\Delta CsVosA5$ 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 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. An asterisk represents p-value <0.05 in T-test analysis when each isolate was compared with the wild type strain (ND90Pr) under constant dark condition.

Table 3. Percentage of different sized (small, normal and large) conidia of wild type (ND90Pr) and knockout mutant ($\Delta CsVosA5$) under different conditions

Strains	Condition	Small	Normal-size	Large
ND90Pr	Constant dark	4%	96%	0%
ND90Pr	Constant light	12%	88%	0%
$\Delta CsVosA5$	Constant dark	29%	39%	32%
$\Delta CsVosA5$	Constant light	14%	38%	48%

Normal conidia were measured at $15\text{-}28 \times 80\text{-}120 \mu\text{m}$ (area value 6000~12000 in SigmaScan software) with 4-8 septa; small conidia were measured at $7\text{-}14 \times 15\text{-}30 \mu\text{m}$ (area value 2000~3000 in SigmaScan software) with 0-1 septa; large conidia were measured at $20\text{-}35 \times 120\text{-}250 \mu\text{m}$ (area value 12000~28000 in SigmaScan software) with 0-8 septa.

Figure1

Figure4

ND90Pr

$\Delta CsVosA5$

Figure8

Figure10

Figure11

Figure12