VeLC Positively Controls Sexual Development in *Aspergillus nidulans*

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Abstract

Fungal development and secondary metabolism is intimately associated via activities of the fungi-specific velvet family proteins including VeA, VosA, VelB and VelC. Among these, VelC has not been characterized in *Aspergillus nidulans*. In this study, we characterize the role of VelC in asexual and sexual development in *A. nidulans*. The velC mRNA specifically accumulates during the early phase of sexual development. The deletion of velC leads to increased number of conidia and reduced production of sexual fruiting bodies (cleistothecia). In the velC deletion mutant, mRNA levels of the brlA, abaA, wetA and vosA genes that control sequential activation of asexual sporulation increase. Overexpression of velC causes increased formation of cleistothecia. These results suggest that VelC functions as a positive regulator of sexual development. VelC is one of the five proteins that physically interact with VosA in yeast two-hybrid and GST pull down analyses. The ΔvelC ΔvosA double mutant produced fewer cleistothecia and behaved similar to the ΔvosA mutant, suggesting that VosA is epistatic to VelC in sexual development, and that VelC might mediate control of sex through interacting with VosA at specific life stages for sexual fruiting.

Introduction

The genus *Aspergillus* is found ubiquitously in our environment and some species are of tremendous importance to humankind as serious human and plant pathogens and as agricultural aids [1]. All *Aspergillus* species commonly reproduce by forming asexual spores called conidia, which are the primary means of infecting host organisms. Conidia also can contain potent allergens and toxic secondary metabolites called mycotoxins [2]. Previous studies proposed that production of some mycotoxins including the most potent natural carcinogens, aflatoxins, is tightly correlated with asexual development (conidiation) [2–4]. *A. nidulans* has served as an excellent model system for studying the mechanisms of asexual development and secondary metabolism [5–7].

The *velvet* family proteins, including VosA, VeA, VelB and VelC, have been identified as key regulators that bridge spore formation and mycotoxin production in *Aspergillus* [8–10]. In addition, some *velvet* proteins form cell-type specific complexes that play differential roles in controlling fungal biology in *A. nidulans* [9,11]. In vegetative cells, the VelB-VeA hetero-complex is required for sexual development and production of the mycotoxin sterigmatocystin (ST) by interacting with LacA [9,12,13]. During conidiation, the VelB-VosA hetero-complex plays a key role in maturation, dormancy and germination of spores [12]. The *velvet* homologues are found in most filamentous fungi and have been reported to regulate development and mycotoxin production in other *Aspergillus* [8,10,14]. In *Aspergillus fumigatus*, VeA represses conidiation, and VelB and VosA control conidial trehalose amount and conidial germination [15,16]. In *Aspergillus flavus*, VeA and VelB are involved in the regulation of conidial production and sclerotia formation [17–19]. VeA also regulates the biosynthesis of secondary metabolites, including aflatoxin in *A. flavus* [20] and *A. parasiticus* [21], gliotoxin in *A. fumigatus* [16], and penicillin in *A. oryzae* [22].

Recent studies have revealed that the *velvet* proteins control various biological processes by acting as transcription factors [23,24]. The conserved *velvet* domain forms a novel DNA-binding motif structurally similar to the Rel homology domain (RHD) of the mammalian transcription factor NF-κB. The *velvet* domain in VosA or the VosA-VelB heterodimer recognizes the specific sequences present in the promoters of developmental regulatory genes and controls their expression [24]. In the human pathogen *Histoplasma capsulatum*, the VosA and VelB orthologues Ryp2 and Ryp3 directly bind to a cis-acting element and activate expression of temperature-responsive target genes [23,25]. These results indicate that the *velvet* proteins are fungal specific transcription factors with DNA-binding activity.

While we now have a better understanding on the roles of three *velvet* regulators VeA, VelB and VosA, the function of VelC
remains unanswered in *A. nidulans*. In this study, we characterized the roles of VelC in regulating development in *A. nidulans*. While the deletion of velC results in reduced cleistothecia production, the overexpression (OE) of velC causes enhanced formation of cleistothecia indicating that VelC is a positive regulator of sexual development. We further show that VosA is epistatic to VelC in *A. nidulans* previously [26–28] and incubated at 37°C with appropriate supplements (simplified as MM) as described [32].

### Materials and Methods

#### Strains and culture conditions

*A. nidulans* strains used in this study are listed in Table 1. Individual strains were grown on solid or liquid minimal medium with appropriate supplements (simplified as MM) as described previously [26–28] and incubated at 37°C. Medium containing sexual development medium pH 6.5: 20 g/l glucose, 1.5 g/l glycine, 0.52 g/l MgSO4.7H2O, 0.52 g/liter KCl, 1.52 g/liter KH2PO4, and 1ml/l of 1000 x trace element solution composed of 22 g/l (NH4)6Mo7O24.4H2O, 50 g/l Na2EDTA; simplified as SM) was used for cleistothecia development test. To determine the numbers of conidia and cleistothecia, wild-type (WT), relevant mutants, and complemented strains were point inoculated and grown on solid MM or SM at 37°C for 4 or 7 days.

To examine the effects of OE of velC by an ectopic copy of velC under the alcA promoter [29,30], all strains were inoculated on solid MM with 0.2% (w/v) ammonium tartrate (MM) and 1 ml/l of 1000 x trace element solution composed of 22 g/l (NH4)6Mo7O24.4H2O, 50 g/l Na2EDTA; simplified as SM) was used for cleistothecia development test. To determine the numbers of conidia and cleistothecia, wild-type (WT), relevant mutants, and complemented strains were point inoculated and grown on solid MM or SM at 37°C for 4 or 7 days.

To examine the effects of OE of velC by an ectopic copy of velC under the alcA promoter [29,30], all strains were inoculated on solid MM with 1% glucose (MMG, non-inducing) or MM with 1% threonine as a sole carbon source (MMT to induce OE of velC) grown on solid MM or SM at 37°C for 7 days. Effects of OE of the velC gene under the niaD [31] promoter in were examined by growing the strains in both MM with 0.2% (w/v) ammonium tartrate (MM + AT, non-inducing) and MMG (inducing, containing 0.6% (w/v) sodium nitrate).

For Northern blot analyses, samples were collected as described [32]. Briefly, for vegetative growth, conidia (5×10⁷ conidia/ml) of WT and mutant strains were inoculated in 100 ml liquid MM in 500 ml flasks and incubated at 37°C. Samples of liquid submersed culture were collected at designated time points, squeezed-dried and stored at −80°C. For sexual and asexual developmental induction, 10 ³ vegetatively grown mycelia were filtered, washed and transferred to solid MM and the plates were air exposed for asexual developmental induction or tightly sealed and blocked from light for sexual developmental induction [32].

Sa. cerevisiae L40 strain was grown on the synthetic dropout (SD) minimal medium with various supplements (10 ml of 100X nutrient solution containing 10 g/l leucine, 2 g/l tryptophan or 2 g/l histidine) [33]. *Escherichia coli* strains, DH5α and BL21 (DE3), were grown in Luria–Bertani medium with ampicillin (50 mg/ml) for plasmid amplification.

#### Generation of the velC mutants

The oligonucleotides used in this study are listed in Table 2. For the deletion of velC, Double-Joint PCR (DJ-PCR) method was used [34]. Both 5’ and 3′ flanking regions of velC were amplified using the primer pairs OMN137;OMN141 and OMN138;OMN142 and *A. nidulans* FGSC4 genomic DNA as a template. The *A. fumigatus* pyrG+ marker was PCR-amplified from *A. fumigatus* AF293 genomic DNA with the primer pair OJH84;OJH85. The DJ-PCR velC deletion construct was amplified with OJH84:ON139:OMN140. The deletion cassette was introduced into RJMP1.59 (Table 1) protoplasts generated by the Vinoflow FCE lysing enzyme (Novozymes) [35]. To generate the double deletion mutants, 5′ and 3′ flanking regions of velC were amplified using OMN54:OHS184 and OMN55:OHS185. The pyrG+ marker was amplified from FGSC4 genomic DNA with the primer pair ONK395:ONK396. After the fusion by DJ-PCR, velC deletion construct was amplified using OMN58;OMN59 and introduced into THS11.1 (Table 1). Multiple (at least three) deletion mutants were isolated and confirmed by PCR followed by restriction enzyme digestion in each case.

To complement ΔvelC, the WT velC locus including its predicted promoter and coding region was amplified with the primer pair OHS178;OHS179, digested with EcoRI and HindIII and cloned into pHS13 [12], which contains p4pyrA [36], a 3xFLAG tag and the trpC terminator [37]. The resulting plasmid pHSN32 was then introduced into the recipient ΔvelC strain THS11.1, in which preferentially a single copy velC+ gets inserted into the pyrA locus complementing the pyrA allele, and gives rise to THS25.1.

To generate the alcA::velC or niaD::velC fusion construct, the velC ORF derived from WT genomic DNA was amplified using

![Table 1. Aspergillus strains used in this study.](image)
| Name     | Sequence (5‘ → 3’)\(^a\) | Purpose       |
|----------|--------------------------|---------------|
| OJA142   | CTGGCAGGTAACAAGTC        | 5’ brlA probe |
| OJA143   | AGAAGTTAACCAGCCTAGA      | 3’ brlA probe |
| OJA150   | CAGTACGCGATATGGAC        | 5’ wetA probe |
| OJA151   | GTGAAGTTGCAAAACAGC       | 3’ wetA probe |
| OJA154   | AGCTCTCTACGATATGGAC      | 5’ aboA probe |
| OJA155   | GTGTGAGATGCCTCAT         | 3’ aboA probe |
| OMN66    | TTCCAGATCCTTGCGAGC       | 5’ vosA probe |
| OMN63    | ATAGAAACAGCACCACCG       | 3’ vosA probe |
| OHS127   | AATTGAAATTGCATGACACCACAGC | 5’ velC probe |
| OHS128   | ATTTAAGCTTCTTCTAATCAGAG  | 3’ velC probe |
| OMN137   | CCGCAAGATCTACAGAGCACAG   | 5’ flanking region of velC |
| OMN138   | TGCAAAACTGGCCAGCTTCTC    | 3’ flanking region of velC |
| OMN140   | TCAAGGGCTAGGTCATTTACC    | 5’ nested of velC |
| OMN141   | GGTGAAGACATATTTGGAGCAG   | 3’ nested of velC |
| OMN142   | AGTGCTCTCTCAGACAGAATA    | 5’ velC with AfupyrG tail |
| OMN54    | TTTCGCCGCTGCTGCAGTA      | 3’ velC with AfupyrG tail |
| OMN55    | AAGAGGGCCTTTGGGCTTCC     | 5’ flanking region of vosA |
| OMN58    | GCTATAACAAAGAGAGAGG      | 3’ flanking region of vosA |
| OMN59    | TTCCGAAAAATATCGCGGCCT    | 5’ nested of vosA |
| OHS184   | ACTTCTGACGTGGAATTGCCCTG  | 3’ nested of vosA |
| OHS185   | TGGTGAACACATGCACACTTGG   | 5’ vosA with pyroA tail |
| OHS186   | GGTGAAAGACATATTTGGAGCAG  | 3’ vosA with pyroA tail |
| OHS167   | AAGTGCCTCTTCTACAGAAGATA  | 5’ vosA with AfupyrG tail |
| OMN304   | CGGGAATTCACGACCCACAGTGGCCTTC | 3’ velC with EcoRI |
| OHS178   | AATTTGGAATTCCTTCACATCTTTGAGAAGGCTCC | 5’ velC with EcoRI |
| OHS179   | AATTTAAGCTTTTCAACTGCGACCTCGAAGATAC | 3’ velC with HindIII |
| ONK114   | TCTATTCCGATGAAAGAATCC    | 5’ pADGal4 |
| ONK115   | TCATAGATCTCTCGCATTAATC   | 3’ pADGal4 |
| OMN329   | CGGGAATTCGATGCGGGCGGAACCTCAG | 5’ vosA with EcoRI |
| OMN330   | ACGGGTGCGCAAGGCCATCAATGAGTGCTAG | 3’ vosA with SalI |
| OMN304   | CGGGAATTCGATGCGGGCGCACTCAG | 5’ velC with EcoRI |
| OMN305   | ATATGGCGGCAGCTTACATCCGAGCTCGAAGAAG | 3’ velC with NotI |
| OMN306   | CGGGAATTCGATGCAAGAGGCCCTTCAGTGGGACCC | 5’ vosA with EcoRI |
| OMN307   | ATATGGCGGCAGCTTACATCCGAGCTCGAAGAAG | 3’ vosA with NotI |
| OMN308   | CGGGAATTCGATGCGGGCGGAACCTCAG | 5’ vosA with EcoRI |
| OMN310   | ATATGGCGGCAGCTTACATCCGAGCTCGAAGAAG | 3’ vosA with NotI |
| OMN313   | CGGGAATTCGATGCGGGCGGAACCTCAG | 5’ vosA with EcoRI |
| OMN314   | ATATGGCGGCAGCTTACATCCGAGCTCGAAGAAG | 3’ vosA with NotI |
| OMN315   | CGGGAATTCGATGCGGGCGGAACCTCAG | 5’ vosA with BamHI |
| OMN316   | ATATGGCGGCAGCTTACATCCGAGCTCGAAGAAG | 3’ vosA with NotI |

\(^a\) Tail sequence is in italic, Restriction enzyme site is in bold.

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the primer pair OMN304;OHS179. The PCR product was then double digested with EcoRI and HindIII and cloned into pHS3, which has the alcA promoter and the trpC terminator [38], or into pH11 that contains the nid promoter and the trpC terminator. The resulting plasmids pHSN7 (alcA::velC) and pHSN12 (nid::velC) were then introduced into FGSC33 and THS11.1, respectively. The velC overexpression (OEvelC) strains among the transformants were screened by Northern blot analysis using a velC ORF probe followed by genomic DNA PCR confirmation for the presence of OE alleles.

**Nucleic acid isolation and manipulation**

To isolate genomic DNA, about 10^6 conidia of WT and mutant strains were inoculated in 2 ml liquid MMG + 0.5% yeast extract, and stationary cultured at 37°C for 24 h. The mycelial mat was collected, squeeze-dried, and genomic DNA was isolated as described [34,39]. Total RNA isolation and Northern blot analyses were carried out as previously described [34,40,41]. To examine the positions of introns, velC cDNA was synthesized from total RNA and sequencing analyses of velC were carried out. The DNA probes were prepared by PCR-amplification of the coding regions of individual genes with appropriate oligonucleotide pairs using FGSC4 genomic DNA as a template (Table 2).

**Yeast two-hybrid assay**

The LexA-based yeast two-hybrid system permitting to detect the LacZ reporter gene expression on the screening plates with X-gal was used. The cDNA of velC coding region was cloned between EcoRI and SalI of pTLexA [42] (kindly provided by Suahn-Kee Chae at Paichai University, Daejeon, Korea), which contains the yeast TRPI selection marker and Zeocin resistance gene. The resulting plasmid pNI39 (bait vector) was introduced into the S. cerevisiae reporter strain L40 (Invitrogen) using lithium acetate-polyethylene glycol-mediated yeast transformation [43]. Then, the A. nidulans cDNA library in pAD-GAL4-2.1 (prey vector; provided by K.-Y. Jahng, Chunbuk University, Jeonju, Korea), which contains the ORF probe followed by genomic DNA PCR confirmation for the velC gene, was screened. The transformants were directly selected on SD plates (-his, -trp, -ura, -leu) with 1 mM 3-amino-1, 2, 4-triazole (3-AT). The big colonies were further transferred to SD plates (-his, -trp, -ura, -leu) with 1 mM 3-AT, 0.2% YE, 2% agar and 500 mg/L Zeocin. The colonies showing intense blue color after incubation were picked. Yeast genomic DNA was isolated from these candidates, and used for transformation of E. coli to recover the prey plasmids by selecting on LB media with ampicillin. Each recovered prey and the pNI39 bait plasmid were further co-introduced back into L40 to confirm they still expressed reporter genes. By direct sequencing of the insert ends of the plasmids of interest with the primer set ONK114 and ONK115 reporter genes. By direct sequencing of the insert ends of the plasmids of interest with the primer set ONK114 and ONK115, the potential VoaA interacting proteins were identified.

**GST pull down**

The voaA cDNA ORF was amplified by the primer pair OMN329;OMN330 using the A. nidulans cDNA library. The resulting amplicon was purified and digested with EcoRI and SalI. The digested voaA amplicon was cloned into pGEX 5X-1 (GE healthcare). The resulting plasmid pNI47 was introduced into E. coli BL21(DE3) to express GST-VoaA. E. coli was grown up to O.D. A600 = 0.5 ± 0.6 at 37°C, 250 rpm, and 0.1 mM IPTG was added for inducing fusion protein expression. The GST fusion protein expression and purification was carried out following the manufacturer's instruction. For concentration and buffer exchange, Amicon Ultra Centrifilter Unit (Millipore) was used. BCA Protein Assay Kit (Pierce) was used to estimate protein concentration.

For expressing voaA interacting (Voa) proteins in vitro, cDNAs of the coding regions of velC, voaA, voaB, voaC or voaD were amplified via RT-PCR and cloned between the EcoRI and NotI (for velC, voaA, voaB and voaD) or BamHI and NotI (voaC) sites in pcDNA3 (Invitrogen) resulting in pNI42, 43, 44, 45 and 46, respectively. pNI42, 43, 44, 45 or 46 was translated in vitro by TNT coupled transcription/translation system (Promega). Briefly, plasmid was incubated with 20 μCi of [35S]-methionine (PE) in TNT mastermix for 90 min at 30°C. Equal amounts of in vitro translated proteins were added to Glutathione bead-GST-VoaA or Glutathione bead-GST (control) suspensions. The mixture was incubated on a mixer at 4°C overnight. After washing three times with lysis buffer, the samples were mixed with Laemmli sample buffer (Bio-Rad) and loaded to SDS-PAGE gel. The gel was dried down under vacuum to 3 layers Whatman 3MM filter paper. Autoradiography was performed at ~80°C with Kodak XAR film (Bio-Rad).

**Microscopy**

The colony photographs were taken by using a Sony digital camera (DSC-F828). Photomicrographs were taken using a Zeiss M² BIO microscope equipped with AxioCam and AxioVision digital imaging software (Zeiss).

**Statistical analysis**

Statistical differences between WT and mutant strains were evaluated with student's unpaired t-test (2-tailed). Mean ± SD are shown. P values <0.05 were considered significant.

**Results**

**Summary of VelC**

The A. nidulans velC gene (EF540816) is composed of a 1,739-bp ORF with one 164-bp intron and predicted to encode a 524-amino-acid polypeptide with a calculated mass of 57.3 kDa (Fig. 1A). To begin to characterize the velC gene, we checked levels of velC mRNA during the lifecycle by Northern blot. As shown in Fig. 1B, the velC transcript is detectable at 48 h of vegetative growth and early phases (24–48 h) of sexual development, but not during asexual development, suggesting that it may play a certain role in sexual development. The predicted VelC protein (ABQ17968) contains one velvet domain in the C-terminal region (252nd–501st aa), which is highly conserved in Aspergillus spp (Fig. 1C). The motif I and motifs 2/3 of the VelC velvet domain are separated by about 100 aa residues. Unlike in other Aspergillus spp., the VelC protein in A. nidulans contains one putative PEST domain (epeSTInd, http://emboss.bioinformatics.nl/cgi-bin/emboss/epestind) which is located between motifs 1 and 2 (392–406 aa).

The deletion of velC increases conidiation

To investigate the role of velC, we generated the velC deletion ΔvelC mutant and complemented strains, and compared their phenotypes. As shown in Fig. 2A, when point inoculated on solid medium and incubated for 4 days, wild type (WT) and complemented strains started to form sexual fruiting bodies, whereas the ΔvelC mutant failed to form cleistothecia. We then compared the numbers of conidia and found that the ΔvelC mutant produced slightly higher number of conidia than WT (data not shown).

To correlate phenotypic changes caused by the absence of velC with the molecular events, we examined the mRNA levels of various asexual development-specific genes including brlA, abdA, velA, and voaA in WT and ΔvelC strains grown under conditions that induce asexual development (Fig. 2B). In WT, accumulation
of brlA mRNA was detectable at 9 h post developmental induction and reduced after 24 h. In ΔvelC strain, however, brlA mRNA started to accumulate at 6 h, stayed at high levels for 9–24 h, and remained clearly detectable even at 48 h. In accordance with brlA mRNA accumulation patterns, levels of abaA, wetA, and vosA mRNA were all higher in the ΔvelC mutant compared to WT. These results indicate that VelC is necessary for the proper control (down-regulation) of assexual developmental regulatory genes.

VelC is necessary for proper sexual development

As enhanced conidiation can result from the reduced sexual development, we addressed the question whether VelC is associated with activating sexual development. WT, ΔvelC, and complemented strains were point-inoculated on SM and incubated in the dark under the air-limited conditions for enhancing sexual fruiting for 7 days. As shown Fig. 3B, the ΔvelC mutant produced significantly reduced number of sexual fruiting bodies compared to WT. 

Figure 1. Summary of velC. (A) Schematic presentation of the velC ORF (shaded box) with an intron (shown by discontinuity in the box). Gene structures were verified by sequence analyses of cDNA of velC. Start codon is assigned as 1 (Top). Domain architecture of the VelC in A. nidulans (Bottom). (B) Northern blot showing level of velC mRNA during the lifecycle of A. nidulans WT (FGSC4). Conidia (asexual spores) were indicated as C. The time (hours) of incubation in liquid submerged culture and post asexual (A) or sexual (S) developmental induction is shown. Equal loading of total RNA was confirmed by ethidium bromide staining of rRNA. (C) Alignment of the VelC protein in Aspergillus spp., A. nidulans (Ani; AN2059), A. fumigatus (Afu; Afu1g09770), A. flavus (Afl; AFL2G_01807), A. oryzae (Aor; AO09000301252), and A. niger (Ani; An04g07320). The conserved motifs are marked by red lines. The PEST domain in A. nidulans VelC was marked by a dotted line. ClustalW (http://align.genome.jp/) and BoxShade 3.21 (http://www.ch.embnet.org/software/BOX_form.html) were used for the alignment.
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Furthermore, the deletion of velC resulted in significantly increased conidia production, and high level accumulation of brlA mRNA even under the conditions favoring for sexual development (Fig. 3B and C). These results suggest that VelC is required for proper sexual development and balanced progression of asexual and sexual development.

Overexpression of velC enhances sexual fruiting

As described above, the deletion of velC resulted in reduced sexual fruiting body production and increased conidiospore production. Two hypotheses regarding the role of VelC can be derived from these results; i) VelC negatively regulates asexual development, which in turn confers sexual development, or ii) VelC positively controls sexual development, which in turn represses conidiation. To address these, we constructed OEvelC strain (alcA(p)::velC) by fusing the velC ORF with the inducible alcA promoter [44]. As shown in Fig. 4A, WT strain exhibited a fluffy phenotype and could not produce sexually developing structures on MMT plates. However, OEvelC strain began to produce Hülle cells (specialized structures supporting sexual fruiting), though did not develop cleistothecia due to the presence of threonine as a sole carbon source, which does not allow sexual development to occur (Fig. 4A). To further examine a potential direct role of VelC in sexual development, the effects of OEvelC under the niiA promoter [31] were examined by growing the individual strains on non-inducing and inducing media. Under non-inducing condition, there were no differences between WT and OEvelC strains in their cleistothecium and conidiospore production. When point inoculated and cultured under inducing conditions, OEvelC strain showed two-fold increased production (p<0.01) of sexual fruiting bodies compared to WT, whereas OEvelC strain produced...
equivalent amounts of asexual spores compared to WT (Fig. 4B&C). Overall, these results strongly support the idea that the controlled expression of velC is necessary for normal fungal development, and that VelC functions as an activator of sexual development.

VelC is one of the VosA interacting proteins

The velvet protein VosA is a multifunctional regulator which plays a complex regulatory role in conidiophore formation and conidia maturation [10,12,45]. VosA forms various complexes including homo-dimer or VelB-VosA hetero-dimer which are localized in the nucleus [12,45]. To better understand the role of VosA, we identified VosA interacting proteins employing yeast-two hybrid assay. The cDNA of VosA (bait) was cloned into pTLexA [42] and the A. nidulans cDNA library in pAD-GAL4-2.1 was screened. After carrying out the procedures to remove the false positive candidates, we identified four VosA interacting (Voi) proteins: VoiA (AN10356), VoiC (AN8795), VoiD (AN4252) and VelC (AN2059) (Table 3). VoiA is a hypothetical protein and contains one BTB/POZ domain, which mediates homomeric or heteromeric dimerization [46]. velC encodes the homolog of μ-1 subunit of clathrin-associated adaptor protein (AP) complex 1, which plays a role in protein sorting in the trans-Golgi network (TGN) and endosomes [47]. VoiD is similar to Histoplasma capsulatum MS8, which is a mold-specific gene required for normal hyphal formation [48]. VelC is one of velvet regulators and contains the velvet domain [10].

To confirm that VosA binds to the Voi and VelC proteins directly in vitro, GST-pull down experiments were carried out. In this experiment, we also added a VoiA similar protein, VoiB (AN0435), which contains one BTB/POZ domain in the N-terminal region. The cDNA of the voi genes was cloned into pcDNA3, and the Voi proteins were translated in vitro and labeled with S35. The vosA ORF was fused with GST in the pGEX 5X-1 vector, and VosA was expressed in E. coli strain BL21 (DE3). Equal amounts of in vitro translated proteins were added to glutathione bead-GST-VosA or glutathione bead-GST (control) suspensions and subjected to pull-down. As shown in Fig. 5, S35 labeled VoiA–D and VelC could be co-purified with GST-VosA, indicating that VosA directly binds to these proteins in vitro.
vosA is epistatic to velC

The above data suggest a possible genetic interaction between VosA and VelC. To address this, we generated the velC and vosA double deletion mutant and compared its phenotypes including asexual and sexual development with the ΔvelC and ΔvosA single mutants. Compared to WT, all three ΔvelC, ΔvosA and ΔvelCΔvosA mutants exhibited defective sexual fruiting under the air-exposed culture condition, and ΔvosA and ΔvelCΔvosA strains produced light-green conidia typical of the ΔvosA mutant (Fig 6A). When point inoculated and cultured for inducing sexual development, the ΔvelC ΔvosA double mutant behaved almost identically to the ΔvosA single mutant, slightly enhanced sexual fruiting compared to the ΔvelC mutant (Fig. 6B). These results suggest that VosA is epistatic to VelC in sexual and asexual development.

Table 3. VosA interacting protein in A. nidulans.

| Gene | ORF( locus) | Annotation |
|------|-------------|------------|
| voiA | AN10356     | Uncharacterized protein (BTB/POZ domain) |
| voiB | AN0435      | Uncharacterized protein (BTB/POZ domain) |
| voiC | AN8795      | AP-1 complex subunit mu-1 |
| voiD | AN4252      | Uncharacterized protein |
| velC | AN2059      | Velvet family protein (velvet domain) |

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cleistothecia in liquid cultures. The velC gene is expressed specifically during the early phase of sexual development. Taken together, we propose that VelC is a sexual activator which acts during the early phase of sexual development.

The VelC homologues have been characterized in A. fumigatus, A. flavus, Penicillium chrysogenum and Fusarium oxysporum [15,17,50,51]. The ΔvelC mutant did not show distinct phenotypes in A. fumigatus [15] and A. flavus [17], suggesting that VelC plays a minor role in asexual development in some Aspergilli. In F. oxysporum, the ΔvelC mutant exhibited increased microconidia production and decreased chromatin accessibility [51]. The velC homologue in P. chrysogenum acts as a repressor of conidiation and activates penicillin biosynthesis [50]. In three fungi, A. nidulans, F. oxysporum and P. chrysogenum, deletion of velC caused increased conidia production, suggesting a potential conserved role of VelC in some fungi.

Studies in F. oxysporum [51] and P. chrysogenum [50] have revealed that VelC physically interacts with other velvet regulators. VelC can interact both with VeA and with VelB in F. oxysporum. The VeA-VelC complex in F. oxysporum plays a negative role in asexual sporulation [51]. In P. chrysogenum, VelC also interacts with VelA or VosA and forms two complexes, which localize in the nucleus. Kopke et al. proposed that one multi-subunit velvet complex regulates penicillin production and conidiation whereas biological roles of two sub-complexes, VelC-VelA and VelC-VosA, are currently unknown [50]. As found in P. chrysogenum, we also identified the A. nidulans VelC protein interacts with A. nidulans VosA in yeast and in vitro (Fig. 5). Most of the phenotypes of the ΔvelC ΔvosA double-deletion mutant, including changes in development, conidial trehalose amount, spore viability and conidial germination, closely resembled those of the ΔvosA single deletion mutant, suggesting that velC is epistatic to vosA in most biological processes.

Collectively, we propose that the velvet proteins or complexes play diverse roles in regulating sexual development in A. nidulans (Fig. 7). We can speculate that the dynamic and differential interaction of velvet, especially VelB, with its partner may be a key determinant of fungal cellular responses. VelB can form VelB-VelB homo-dimer, VelB-VosA, or VelB-VeA heterodimers [12,45]. In hyphae, VelB mainly interacts with VeA and forms the VelB-VeA heterodimers which is required for the initiation,

Discussion

The velvet family proteins play vital roles in development and secondary metabolism in filamentous fungi [8]. While VeA, VelB and VosA have been characterized in A. nidulans, the role of VelC was unclear. In this study, we present the experimental evidence that VelC plays a vital role in controlling asexual and sexual development in A. nidulans. The ΔvelC strain exhibited enhanced production of conidiospores in conjunction with the reduced formation of sexual fruiting bodies (Figs. 2 & 3). Furthermore, examination of mRNA levels of asexual developmental genes suggests that VelC is required for the proper control of auxsexual specific genes. We then asked whether VeC acts as a repressor of asexual development or an activator of sexual development. First, we examined the phenotypes of the ΔvelC mutant and found that the ΔvelC mutant cannot produce conidiophores and induce velC expression in liquid submerged culture (data not shown). Second, OE of velC causes elevated production of sexual fruiting bodies. These results indicate that VelC may function as a sexual activator, which indirectly represses conidiation in A. nidulans. Unlike OE of vel [49], however, OE velC strain could not form cleistothecia in liquid cultures. The velC gene is expressed

Figure 5. VelC physically interacts with VosA. GST pull down assay for GST or GST-VosA and in vitro translated 35S-VosA interacting proteins. The in vitro translated proteins were divided into two parts (each 20 µl) and mixed with GST alone (right panel) or the GST-VosA protein (left panel). The expected protein size of VosA, VosB, VelC, VosD or VoiC is about 38, 31, 62, 20.9 and 49 kDa, respectively.

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Figure 6. Double mutant analysis. (A) Colony photographs of WT (FGSC4), ΔvelC (THS11.1), ΔvosA (THS15.1) and ΔvelC ΔvosA (THS26.1) strains grown on solid MM for 4 days. (bar = 0.5 mm). (B) Quantitative analysis of conidiation of strains shown in (A) (** P<0.01; *** P<0.001).

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progression and completion of sexual development [12]. Some VelB-VosA hetero-complexes may also exist in hyphae. During early phase of sexual development, VelC is produced, which then physically interacts with VosA. Such VelC-VosA interaction leads to increased formation of the VelB-VeA hetero-complex, which then triggers the sexual fruiting process. The VelC protein may also play a potential role in activating sexual development. In ascospores, the VelB protein mainly interacts with VosA [12] and forms the VelB-VosA complex which may play a critical role in regulating trehalose biogenesis and ascospore viability. Further studies revealing the molecular mechanisms of VelC-mediated developmental control will provide novel insights into complex fungal biology.

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Author Contributions

Conceived and designed the experiments: H-SP S-CK J-HY. Performed the experiments: H-SP T-YN. Analyzed the data: H-SP T-YN K-HH. Contributed reagents/materials/analysis tools: K-HH S-CK J-HY. Wrote the paper: H-SP J-HY.

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