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Neurospora crassa ve-1 affects asexual conidiation

Özgür Bayram [a], Sven Krappmann [a], Stephan Seiler [a,b], Nico Vogt [a], Gerhard H. Braus [a,*]

[a] *Institute of Microbiology & Genetics, Department of Molecular Microbiology and Genetics, Georg-August-University, Grisebachstr. 8, D-37077 Göttingen, Germany*
[b] *DFG Research Center for Molecular Physiology of the Brain (CMPB), D-37077 Göttingen, Germany*

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Abstract

The *velvet* factor of the homothallic fungus *Aspergillus nidulans* promotes sexual fruiting body formation. The encoding *veA* gene is conserved among fungi, including the ascomycete *Neurospora crassa.* There, the orthologous *ve-1* gene encodes a deduced protein with high similarity to *A. nidulans* VeA. Cross-complementation experiments suggest that both the promoter and the coding sequence of *N. crassa ve-1* are functional to complement the phenotype of an *A. nidulans* deletion mutant. Moreover, *ve-1* expression in the heterologous host *A. nidulans* results in development of reproductive structures in a light-dependent manner, promoting sexual development in the darkness while stimulating asexual sporulation under illumination. Deletion of the *N. crassa ve-1* locus by homologous gene replacement causes formation of shortened aerial hyphae accompanied by a significant increase in asexual conidiation, which is not light-dependent. Our data suggest that the conserved *velvet* proteins of *A. nidulans* and *N. crassa* exhibit both similar and different functions to influence development of these two ascomycetes.

Keywords: Ascomycete; Conidiation; Fruiting body; *Velvet*

1. Introduction

Light is an essential source of energy that drives life on earth. Animals, plants, fungi, and cyanobacteria exploit the various spectra of light in order to survive. Light can cause many behavioural and developmental changes in metabolism, growth, directionality, or morphogenesis, respectively (Gyula et al., 2003). In addition, light is a trigger for adjusting diurnal duration or circadian rhythms, which can be found from cyanobacteria to fungi to humans (Bell-Pedersen et al., 2005). Among living organisms, fungi occupy an important position to study light and its effects on development in a straight-forward and effortless manner. Light-driven experiments with different groups of fungi have demonstrated that fungi are responsive to a broad spectral range of light, among which blue and red light display prominent effects on fungal growth (Mooney and Yager, 1990; Kües, 2000; Cerda-Olmedo, 2001; Liu et al., 2003; Idnurm and Heitman, 2005; Purschwitz et al., 2006).

Two model organisms, *Neurospora crassa* and *Aspergillus nidulans*, which belong to the ascomycetes, are distantly related fungi that exhibit different responses to the light spectrum. *N. crassa* is more receptive to blue light while *A. nidulans* responds primarily to red light illumination (see below). Both fungi are suitable to study molecular mechanisms leading to development and cell differentiation. They are predominantly haploid during their life cycle, which makes them preferable to investigate and observe the interaction between genes and development. In submerged culture, *N. crassa* and *A. nidulans* produce a web of branched vegetative hyphae called mycelium. When exposed to an aerial interface, mycelia differentiate and form asexual spores termed conidia. Two types of asexual spores are produced by *N. crassa*: multinucleate macroconidia and uninucleate microconidia (Siegel et al., 1968; Springer, 1993; Maheshwari, 1999). Conidiation is induced primarily by dessication, carbon source limitation

* Corresponding author. Fax: +49 0 551 393330.
E-mail address: gbraus@gwdg.de (G.H. Braus).

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as well as light and changes in CO_2 partial pressure. A phlethora of conidia-specific genes has been described (reviewed by Springer and Yanofsky, 1989). In *A. nidulans*, mycelia grown on a solid and illuminated surface develop asexual spore-producing structures called conidiophores. This is initiated by a foot cell that swells and gives rise to layers of metulae, which in turn result in the phialide cells producing chains of asexual conidia by consecutive budding (Adams et al., 1998). Every stage of this conidiophore formation is controlled by temporal and spatial expression of transcription factors that are specific for each developmental stage, such as BrlA, AbaA, and WetA (Adams et al., 1988; Marshall and Timberlake, 1991; Andrianopoulos and Timberlake, 1994).

Sexual differentiation in ascomycetes results in fruiting bodies of different shapes, depending on the taxonomical group. The homothallic fungus *A. nidulans* forms sexual fruiting bodies called cleistothecia that are spherical, closed structures enveloping the asci (reviewed by Braus et al., 2002). *N. crassa* produces bottle-shaped sexual architectures named perithecia, and sexual development of this fungus is controlled by two mating type genes (Pöggeler et al., 2006). Nitrogen limitation causes the formation of protoperithecia which serve as recipient for male nuclei of the opposite mating type, and fertilized protoperithecia develop into perithecia in which eight linearly ordered homokaryotic ascospore containing asci are formed. Sexual development in *A. nidulans* can also occur either by mating of two strains but also *via* selfing in the absence of a partner. In this ascomycete, sexual development takes place following asexual sporulation and is initiated by the formation of specific mycelial aggregates formed by so-called Hülle cells. Within these nests, the spherical products of sexual differentiation develop that contain meiotically derived, red-pigmented ascospores (Braus et al., 2002; Sohn and Yoon, 2002).

Developmental programmes such as spore formation are often subject to various environmental stimuli, among them illumination. Especially blue light responses of *N. crassa* have been widely studied: blue or UV light stimulates carotenoid synthesis, induction of protoperithecia and their prototropism, asexual spore formation, hyphal growth induction and entrainment of the circadian clock (Linden et al., 1997). Many of these responses are regulated through the phototropin-like proteins WHITE COLLAR-1 (WC-1) and WHITE COLLAR-2 (WC-2), the former being the first fungal photoreceptor described (Ballario et al., 1996; Linden and Macino, 1997; Froehlich et al., 2002; He et al., 2002). A second type of blue light receptor called VIVID (VVD), which contains a sensory light–oxygen–voltage (LOV) domain that binds flavins like FAD or FMN, is presumed to sense light intensity changes (Crosson et al., 2003; Schwerdtfeger and Linden, 2003).

In contrast, *A. nidulans* shows a distinct red light response. For *A. nidulans*, illumination negatively affects fruiting body formation as it inhibits sexual development and promotes the asexual developmental programme. When incubation is carried out in the dark, increased numbers of fruiting bodies are formed; on the other hand, growth in day light conditions or under red light illumination leads to asexual sporulation (Zonneveld, 1977). Recently, a phytochrome red light receptor encoded by the *fphA* gene was shown to be responsible for repression of sexual fruiting body formation and induction of conidiation under red light conditions (Blumenstein et al., 2005). The so-named *velvet* factor, firstly described by Käfer (1965), plays a prominent role in light-dependent development of *A. nidulans*: *veA1* mutants show retarded sexual development and excessive asexual sporulation. Later it was demonstrated that in a *wild-type* veA^{+} background red light promotes the formation of conidiospores, while *veA1* strains are not affected by this kind of illumination (Mooney and Yager, 1990). Therefore, the *veA* gene product was proposed to be a negative regulator of asexual conidiation whose function could be suppressed by red or day light (Champe et al., 1981; Mooney and Yager, 1990; Timberlake, 1990; Yager, 1992). The encoding *veA* locus was cloned and preliminary characterized, demonstrating that deletion of the gene results in an acleistothecial phenotype even under conditions that promote sexual development (Kim et al., 2002). Accordingly, *veA* over-expression results in Hülle cell formation even in submerged culture. This work also demonstrated that the *veA1* allele was formed as a result of G-to-T nucleotide substitution in the start codon of the *wild-type veA* gene leading to downstream translational initiation and synthesis of a truncated gene product. Moreover, VeA's role seems to be not only restricted to sexual development in *Aspergillus* species, as it was also shown that the *veA* gene product of *A. parasticus* affects sclerotial production as well as secondary metabolism (Calvo et al., 2004) and that a *veA* gene disruption in the asexual pathogen *A. fumigatus* reduces asexual sporulation in dependency of the nitrogen source (Calvo et al., 2004; Krappmann et al., 2005). Moreover, recent data suggest that the *Fusarium verticilloides* orthologue FvVE1 regulates filamentous growth and the ratio of microconidia to macroconidia, and that *velvet*-like proteins are conserved throughout the fungal kingdom (Li et al., 2006).

The genome sequences of *N. crassa* and *A. nidulans* have been determined (Galagan et al., 2003, 2005), and inspection of both genomes reveals many orthologous sequences, the function of whose may be conserved or altered during the course of evolution. In this report we show that the *N. crassa* genome contains one copy of a *velvet* gene orthologue encoding a protein that shares 50% similarity to that of *A. nidulans*, VeA. The locus was targeted *via* homologous gene replacement to uncover that deletion of the *N. crassa ve-1* gene results in shortened aerial hypha formation accompanied by increased conidiation, which can be complemented by re-introduction of a 7 kb genomic fragment comprising *ve-1*. Cross-complementation experiments indicate that the *N. crassa ve-1* gene rescues the phenotype of an *A. nidulans veAΔ* deletion strain and that it is

regulated in the same manner like *veA* in the exogenous host, supporting a functional as well as structural conservation of the *velvet* factor among these two ascomycetes.

2. Materials and methods

2.1. Strains, media, and growth conditions

Fungal strains used during this study are listed in Table 1. *N. crassa* strain FGSC987 (*74-OR23-1A*) served as recipient for the *ve-1* deletion cassette and for over-expression experiments; *A. nidulans* strain DVAR1 was used for cross-complementation experiments. Standard laboratory *Escherichia coli* strains DH5α and MACH-1 (Invitrogen) were used for preparation of plasmid DNA and were propagated in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) supplemented with 100–150 μg ml^{-1} ampicillin. Minimal medium (0.52 g l^{-1} KCl, 0.52 g l^{-1} $MgSO_4$, 1.52 g l^{-1} KH_2PO_4, and 0.1% trace element solution, pH 6.5) was used for growth of *A. nidulans* strains (Käfer, 1965), supplemented with appropriate amounts of 4-aminobenzoic acid (PABA, 1 μg ml^{-1}), nourseothricin-dihydrogen sulfate (100–120 μg ml^{-1}) (clonNAT, Werner BioAgents), pyrithiamine (Takara Bio Inc.) (0.1 μg ml^{-1}); 1% D-glucose was used as the source of carbon together with 10 mM ammonium or nitrate as nitrogen source. *N. crassa* strains were grown in either liquid or solid (supplemented with 2% agar) Vogel's minimal media with 1.5% sucrose, and as selective agents nourseothricin-dihydrogen sulfate (35 μg ml^{-1}) (clonNAT, Werner BioAgents) or hygromycin (Invivogen) (200 μg ml^{-1}) were added. SC media was prepared as described by Davis and DeSerres (1970), composition of cornmeal agar media was 2% cornmeal agar (Sigma) containing 0.1% glucose. For light response experiments, a portable self-made illumination chamber equipped with blue and red lights of 470 nm and 630 nm wavelength, respectively, was used.

2.2. Transformation procedures

Escherichia coli cells were transformed using calcium- and manganese-treated cells (Hanahan et al., 1991). *A. nidulans* was transformed by polyethylene glycol-mediated fusion of protoplasts described in detail by (Punt and van den Hondel (1992). Transformation of *N. crassa* spheroplasts was performed essentially as described earlier by Vollmer and Yanofsky (1986).

2.3. Recombinant DNA procedures and hybridisation techniques

For recombinant DNA technology, standard protocols were performed (Sambrook et al., 1989). Taq, Pfu (MBI Fermentas) and, for long templates, Platinum Taq DNA high fidelity polymerase (Invitrogen) were used in polymerase chain reactions (PCRs, Saiki et al., 1986), and crucial cloning steps were validated by sequencing on an ABI Prism 310 capillary sequencer (PE Biosystems). Fungal genomic DNAs were prepared from ground mycelia (Kolar et al., 1988), and hybridisation analyses using nylon membranes were conducted essentially as described (Southern, 1975) using the OZG20/32-amplified *ve-1* 5′ UTR flanking region as a probe. Total RNA samples were purified using the TRIzol reagent (Invitrogen) followed by Northern hybridization according to the protocols cited by Brown and Mackey (1997). The Stratagene Prime-It II kit was utilized to radioactively label hybridization probes in the presence of [α-^{32}P]dATP (Feinberg and Vogelstein, 1983). To produce autoradiographies, washed membranes were exposed to Kodak X-Omat films. Quantification of signal intensities was carried out employing a Bio-Imaging Analyzer from Fuji Photo Film Co. Ltd. Sequence data were analysed using the Lasergene software package from DNAStar, and protein sequence alignments were created by the Clustal W method (http://www.ebi.ac.uk/clustalw/index.html; Thompson et al., 1994). Mapping and alignment of promoter regions for transcription factor binding sites was performed *via* the web server http://genome.imim.es/software/meta/meta.html (Blano et al., 2006).

2.4. Plasmid constructions

The plasmid constructs created and used throughout this study are listed in Table 2; oligonucleotide sequences are given in Table 3.

Table 1
Fungal strains used in this study

Strain	Genotype	Reference
Neurospora crassa		
Wild-type	FGSC987 (74-OR23-1A)	Fungal Genetics Stock Center
NCOB1	*ve-1Δ::hph*	This study
NCOB2	^{p}*ve-1::ve-1::ve-1^{t}*, ^{p}*gpdA::nat^{R}*, ve-1Δ::hph*	This study
Aspergillus nidulans		
DVAR1	*pabaA1, yA2; argBΔ::trpC; trpC801; veAΔ::argB*	Kim et al. (2002)
AGB285	^{p}*veA::ve-1::veA^{t}*, *ptrA; pabaA1, yA2; argBΔ::trpC; trpC801, veAΔ::argB*	This study
AGB286	^{p}*ve-1::veA::ve-1^{t}*, ^{p}*gpdA::nat^{R}*; pabaA1, yA2; argBΔ::trpC; trpC801, veAΔ::argB*	This study
AGB287	^{p}*ve-1::ve-1::ve-1^{t}*, ^{p}*gpdA::nat^{R}*; pabaA1, yA2; argBΔ::trpC; trpC801, veAΔ::argB*	This study

Table 2
Plasmid constructs employed in this study

Plasmid	Description and characteristics	Reference
pMP6	*^{p}cpc-1::hph::trpC^{t}, bla*	Seiler et al. (2006)
pBluescript II KS	General cloning plasmid [*bla*, multiple cloning site]	Stratagene
pPTRII	Autonomously replicating *Aspergillus* plasmid [*ptrA, AMA1, bla*]	Takara
pNV1	*nat^{R}* plasmid conferring resistance for clonNAT	Seiler et al. (2006)
pME3174	*ve-1* deletion cassette [*ve-1::hph*]	This study
pME3175	7.4 kb *ve-1* genomic locus (SspI) subcloned in EcoRV of pBluescript II KS	This study
pME3176	7.2 kb *ve-1* genomic locus in ApaI site of pNV1	This study
pME3177	5 kb *ve-1* locus in ApaI site of pNV1	This study
pME3178	4.6 kb *veA* HindIII genomic locus with *ptrA* (in SmaI) resistance in pBluescript II KS	This study
pME3179	chimeric *^{p}veA::ve-1::veA^{t}* construct	This study
pME3180	chimeric *^{p}ve-1::veA::ve-1^{t}* construct	This study

Table 3
Oligonucleotides utilized in this study

Designation	Sequence	Restriction site
OZG17	5′-*ggccactcaggcc* GAA TTC AAC TGT CCG ATA TCG GTA-3′	SfiI
OZG18	5′-*ggcctagatggcc* TCT AGA AAG AAG GAT TAC CTC TAA AC-3′	SfiI
OZG19	5′-GTC GAC GAC TTT GTT GGT TGG CAT TCA CCG-3′	
OZG20	5′-*ggcctgagtggcc* GGT TGA TCA TTT TTA CAC GAC GGT-3′	SfiI
OZG21	5′-*ggccatctaggcc* CCT GTT CAC CTC ACA ACC AAA TGG-3′	SfiI
OZG22	5′-*gtcgac* CGG CAC TTA CAG TAT CCT CCG GTA-3′	SalI
OZG32	5′-CTT CAA AGG GCT CTC TGA GGT TGG CT-3′	
OZG36	5′-*agatct* TCA ATA CCC GCC AAT ATC TGC CTG-3′	BglII
OZG37	5′-*tctaga* ATG GGT GCT CAG GTT ATC GCC GCT-3′	XbaI
OZG47	5′-*gggccc* TCG TCG AAC CTA ATA AGT GGT AG-3′	ApaI
OZG48	5′-*gggccc* GTC CCT TGA TAA ACG TTC CCT GG -3′	ApaI
OZG49	5′-*actagt* ATG GCT ACA CTT GCA GCA CCA CCA-3′	SpeI
OZG50	5′-*cacgtg* TAA CGC ATG GTG GCA GGC TTT GAG A-3′	PmiI

A 5′ UTR flanking region of the *N. crassa ve-1* gene was amplified with OZG21/22 and cloned into the SmaI site of pBluescript KS II (Stratagene), and the OZG19/20-amplified 3′ UTR of *ve-1* was cloned into the EcoRV site of this plasmid. Finally, the *hph* marker gene conferring hygromycin resistance was amplified using pMP6 as template with OZG17/18, digested with SfiI and inserted into the SfiI site between the flanking regions yielding pME3174. A 7.4 kb genomic fragment (SspI) of the *N. crassa ve-1* locus was cloned in EcoRV of pBluescript KS II giving pME3175; from this, a 7.2 kb fragment was released with ApaI and inserted into pNV1 (ApaI) to yield pME3176, which was used for complementation of NCOB1. The 5 kb genomic fragment of *ve-1* of *N. crassa* was amplified with OZG47/48, digested with ApaI and ligated into the ApaI site of pNV1 to result in pME3177. To create the pME3179 chimeric plasmid, pME3178 was digested with XbaI and BglII to remove the *veA* coding sequence, and the OZG36/37-amplified, XbaI–BglII digested *ve-1* ORF was inserted yielding the *ve-1* gene driven from the *A. nidulans veA* promoter. The pME3180 chimeric construct was generated by digesting pME3177 with SpeI/PmiI and inserting OZG49/50-amplified and SpeI/PmiI-digested cDNA of *veA*, which had been amplified from the sexual stage-specific cDNA plasmid library pCNS4 (Krappmann et al., 2006).

3. Results

3.1. Neurospora crassa VELVET-1 displays high similarity to A. nidulans VeA

The heterothallic ascomycete *N. crassa* is known for its response to blue light, which is controlled by the protein WC-1. In the homothallic ascomycete *A. nidulans*, however, a developmental response to red light is more pronounced: illumination with red light inhibits sexual development and induces asexual conidiation through phytochrome-mediated signal transduction (Blumenstein et al., 2005), which presumably involves the *veA* gene product. The established roles of VeA in *A. nidulans* development raised the question if there is any *veA*-like gene in *N. crassa* and what roles it may perform in the *N. crassa* light response.

Inspection of the *N. crassa* protein database (www.broad.mit.edu/annotation/genome/neurospora/Home.html) disclosed one protein orthologous to *A. nidulans* VeA encoded by the locus NCU01731.2. According to the automatic annotation, the locus contains two exons interrupted by one intron located in the first half of the gene with the capacity to encode a protein of 555 amino acids. This deduced protein displays a high degree of similarity to VeA of *A. nidulans* (similarity index of 49.5%) and other VeA-like proteins of various *Aspergillus* species (Fig. 1a).

Accordingly, the identified gene of *N. crassa* was designated *ve-1*. When aligning VE-1 with VeA by the EMBOSS Pairwise Alignment Algorithm (http://www.ebi.ac.uk/emboss/align/index.html), many conserved amino acid substitutions are noticable. VeA contains a putative nuclear localization signal (NLS) sequence in its N-terminus (Kim et al., 2002; Stinnett et al., 2007) and a putative nuclear export signal (NES, http://www.cbs.dtu.dk/services/NetNES/) in the middle of the protein sequence, while VE-1 apparently does not comprise any classical NLS or NES motif. VeA also contains a so-named PEST (Pro, Glu or Asp Ser, Thr-rich) box responsible for protein degradation (Rechsteiner, 1990; Kim et al., 2002); when submitting the VE-1 sequence to the PEST finder internet tool (https://emb1.bcc.univie.ac.at/toolbox/pestfind/pestfind-analysis-webtool.htm), one putative PEST motif (K^{172}DPEEPNAPPDGSPGSFDF^{191}) could be identified for this protein. Moreover, highly conserved patterns in the N-termini of both proteins exist, which might indicate a specific function of the N-terminus. This is supported by the *A. nidulans veA1* allele, which encodes an N-terminal-truncated protein and results in reduced levels of sexual development (Mooney et al., 1990). Transcription factor map alignment based on the upstream regions of both genes demonstrate that conserved stretches along the 5′ untranslated regions (5′ UTRs) can be detected (Fig. 1b), and suggests similar elements conferring transcriptional regulation of both genes.

b

C	TF	veA positions	ve-1 positions
	V$MYB_Q3	10-15	12-17
	V$STAT5A_04	173-180	132-139
	V$STAT5A_04	276-283	178-185
	V$STAT3_02	287-294	189-196
	V$CAP_01	308-315	210-217
	V$STAT3_02	335-342	233-240
	V$CAP_01	382-389	289-296
	V$TEF1_Q6	432-437	308-313
	V$GATA2_01	494-503	384-393
	V$STAT5A_04	574-581	447-454
	V$MZF1_01	589-596	458-465
	V$ETF_Q6	628-634	494-500
	V$STAT6_02	658-665	512-519
	V$ETS_Q4	666-671	522-527
	V$FOXM1_01	723-731	597-605
	V$STAT6_02	762-769	639-646
	V$GATA6_01	783-792	750-759
	V$CAP_01	822-829	829-836
	V$EN1_01	844-850	852-858
	V$CDXA_02	945-951	942-948
	V$CAP_01	955-962	952-959

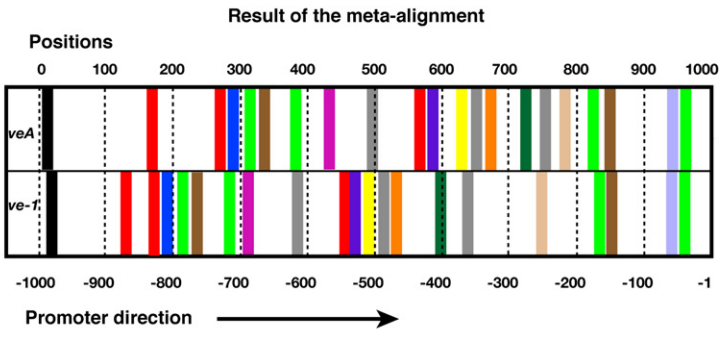

Fig. 1. Velvet proteins are evolutionary conserved in filamentous fungi. (a) Clustal W alignment of *velvet* proteins from *Aspergillus* species and *Neurospora crassa* with conserved residues shaded in black. The proteins comprise several highly conserved motifs in their N-termini, shown as large gray-dashed rectangle. *A. nidulans* VeA bears a bipartite nuclear localization signal (NLS) motif (white rectangle and plus symbol), and all VeA orthologues contain a putative PEST (Pro, Ser, Asp, and Thr) motif in different positions of the proteins (gray rectangles). *A. nidulans* VeA also bears a potential nuclear export signal (NES) indicated as thick black bar and stars. *N. cra*: *N. crassa* VE-1; *A. par*: *A. parasiticus* VeA; *A. fum*: *A. fumigatus* VeA; *A. nid*: *A. nidulans* VeA. (b) Result of TF-map alignment (Blano et al., 2006) considering 1000 nucleotides upstream of the *veA* and *ve-1* coding sequence, respectively. TF-maps were created using TRANSFAC matrices at a threshold of 0.75 to result in 190 elements for the *veA* and 149 elements for the *ve-1* upstream region. The deduced TF-map alignment, containing 21 elements, is schematically presented on the right hand side, transcription factors with coordinates of respective binding sites are listed in the left hand side table (C, colour; TF, transcription factor designation).

3.2. Deletion of ve-1 results in increased conidiation associated with stunted aerial hyphae

Observing the high degree of conservation of the *N. crassa ve-1* gene product made it tempting to address the cellular function of this protein in order to answer the question if it has conserved functions in *N. crassa*. For that aim, we constructed a deletion module containing a hygromycin resistance cassette flanked by 2.5 kb upstream and downstream regions of *ve-1*. The construct was transformed into the recipient *N. crassa* strain FGSC987 (*74-OR23-1A*), and a homologous gene replacement event was screened by Southern hybridization using a probe complementary to the 5′ UTR region of *ve-1* (Fig. 2a and b). As a result, three transformants could be confirmed to carry the desired gene replacement.

These deletion strains were backcrossed with the *wild-type* strain in order to remove secondary genetic defects and were screened for their normal developmental stages. All three deletion strains were identical in phenotypic appearance, therefore one representative, strain NCOB1, was investigated further. This *ve-1Δ* deletion strain displays various phenotypic defects: an increase in asexual sporulation associated with reduced aerial hyphae formation on Vogel's minimal medium (Fig. 2c and d). In addition, its hyphal branching pattern appears slightly disturbed (not shown). The deletion strain's growth rate is reduced by 15%, which is probably due to the hyphal branching distur-

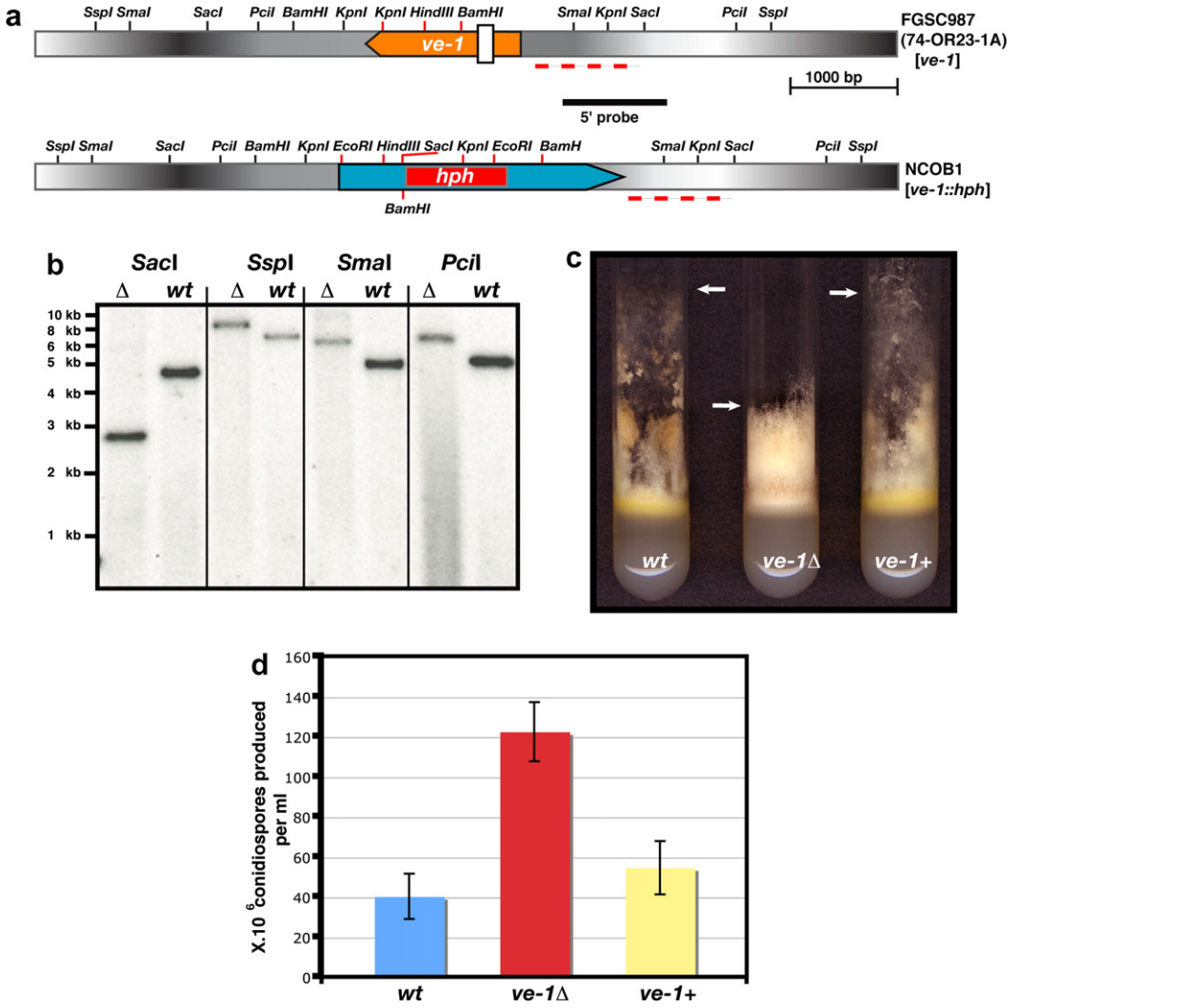

Fig. 2. Deletion of *ve-1* results in increased conidiation associated with stunted aerial hyphae formation. (a) Comparative depiction of the *wild-type ve-1* locus in strain FGSC987 (*74-OR23-1A*) and the *ve-1Δ::hph* locus in the knock-out strain NCOB1. The black bar indicates the region encompassed by the probe employed in Southern analyses. (b) Autoradiography results of Southern hybridization validates the homologous gene replacement [sizes of detected restriction fragments as deduced from gene loci maps: SacI: 2.9 kb for deletant (Δ), 4.9 kb for *wild-type* (wt); SspI: 9.1 (Δ) vs. 7.5 kb (wt); SmaI: 6.8 (Δ) vs. 5.2 kb (wt); PciI: 7.2 (Δ) vs. 5.6 kb (wt); sizes of length standard fragments were extrapolated from the initial agarose gel electrophoresis image]. Phenotypical investigation of the *N. crassa ve-1Δ* strain unveils shortened aerial hypha formation (c) and increased asexual conidiospore formation (d); both defects are complemented by re-transformation with the *ve-1* genomic locus. White arrows in (c) indicate the edge of upwards growing hyphae. Strains were grown at room temperature for 5 days, conidiospores were quantified by counting spores of four tubes for each of strain; standard deviations are indicated as black bars.

bance. The increase in conidiation was quantified by counting the spores produced in slants to be almost three fold more than in FGSC987 (*74-OR23-1A*) (Fig. 2d). All phenotypes, aerial hyphae and asexual development abnormalities, could be complemented by introducing 7 kb genomic fragment comprising the *ve-1* gene locus. The deletion strain was also investigated for its ability for sexual propagation, to reveal that it is both female and male fertile producing ascospore-containing perithecia on cornmeal agar to the same extent as the *wild-type N. crassa* progenitor strain. One additional phenotype, which is blocked protoperithecia development on synthetic crossing (SC) media, could, however, not be complemented by the 7 kb genomic fragment (data not shown). The fact that the mutant is able to form protoperithecia on cornmeal medium but not on the defined synthetic one is elusive but may be based on the complex nature of the former growth substrate. The deletion strain did not reveal any abnormality under different light sources. These findings suggest that VE-1 is important for the regulation of hyphal growth, morphology and repression of asexual sporulation in *N. crassa*, but differences in sexual development on two tested media may imply the existence of some redundantly acting gene products during sexual development in *N. crassa*.

3.3. ve-1 is transcribed constitutively during different life stages of N. crassa

Temporal and spatial expression of conserved regulators determine the developmental fate of related organisms. The expression of *veA* in *A. nidulans* is regulated during the course of distinct developmental stages: during vegetative growth it is transcribed at basal levels, whereas transcript levels intensify during asexual development and become stronger for the period of sexual development (Kim et al., 2002).

Accordingly, we considered that there might be a similar regulation of *ve-1* expression in *N. crassa*. Mycelia of the vegetative growth phase was grown in Vogel's minimal media for 15 h under constant shaking. Asexual development was triggered in Vogel's minimal cultures grown for 5 days without shaking to induce conidiation, and sexual development was induced by mycelial growth for three days in liquid SC media followed by incubation on SC plates for additional five days. Northern hybridisation experiments with RNA preparations from these different developmental stages revealed that, in contrast to *veA* expression in *A. nidulans*, *ve-1* appears to have a constitutive transcription pattern throughout the probed developmental stages of *N. crassa* (Fig. 3a), indicating a general importance of the *ve-1* gene during all life phases of the organism and not only during one-specific stage.

Aspergillus nidulans and *N. crassa* exhibit red and blue light responses, respectively. Thus we asked whether *ve-1* expression is regulated by different wavelengths emitted from light sources. Cultures *N. crassa* were grown without shaking at 30 °C for 2 days under white, blue, and red light illumination, respectively, or in the darkness and analyzed for the expression of *ve-1* by Northern blot hybridisation (Fig. 3b). Inspection of the resulting autoradiographies revealed that, when compared to dark and white light conditions, steady-state transcript levels of *ve-1* increase under red light, indicating a light-dependent regulation of *ve-1* expression.

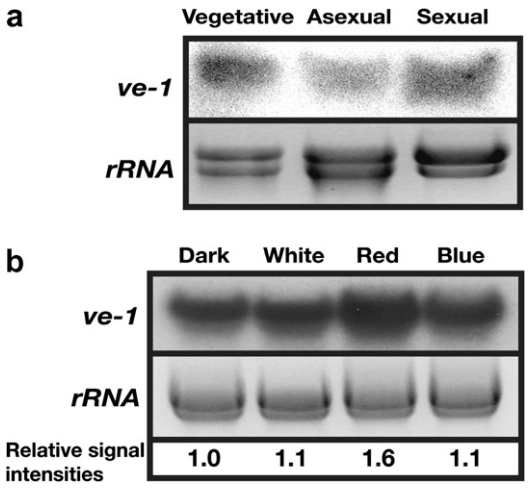

Fig. 3. *ve-1* expression is constitutive during development and increases upon red light illumination. (a) Northern hybridization of *ve-1* displays constant expression levels during vegetative growth (15 h, in Vogel's liquid minimal media culture), asexual (5 days in Vogel liquid minimal media without shaking) and sexual development on SC (three days in synthetic crossing liquid media, and subsequently plated on SC plates for additional 5 days). (b) For light-dependent expression analysis, *ve-1* expression was monitored under illumination by different light sources in Vogel's minimal media grown at 30 °C for two days. Northern hybridisation intensities of *ve-1* under red light conditions are elevated by 60–70%, as quantified from autoradiographies obtained from two-independent experimental setups.

3.4. VE-1 and the ve-1 promoter are functional in A. nidulans

To test, if the two *velvet* proteins are functionally conserved among these distantly related filamentous ascomycetes, we expressed the *N. crassa* protein VE-1 in *A. nidulans* to test whether it is a functional orthologue of VeA, and to monitor its regulation in the heterologous host. For that purpose, we constructed a set of chimeric expression constructs. One plasmid (pME3179) contains the *ve-1* coding sequence driven by the *A. nidulans veA* promoter and terminator sequences to test functionality of the *ve-1* coding sequence in *A. nidulans.* This construct was transformed into the *veAΔ* deletion strain DVAR1 to yield AGB285. While a *wild-type A. nidulans* strain develops cleistothecia after four to five days at 37 °C, AGB285 needed about 8–9 days under identical incubation conditions to produce fruiting bodies that bear meiotically derived, fertile ascospores (Fig. 4). In addition to this delayed complementation of the sexual development defect, a brown pigmentation, which is a prominent phenotype caused by the *veAΔ* deletion, was absent (Fig. 4).

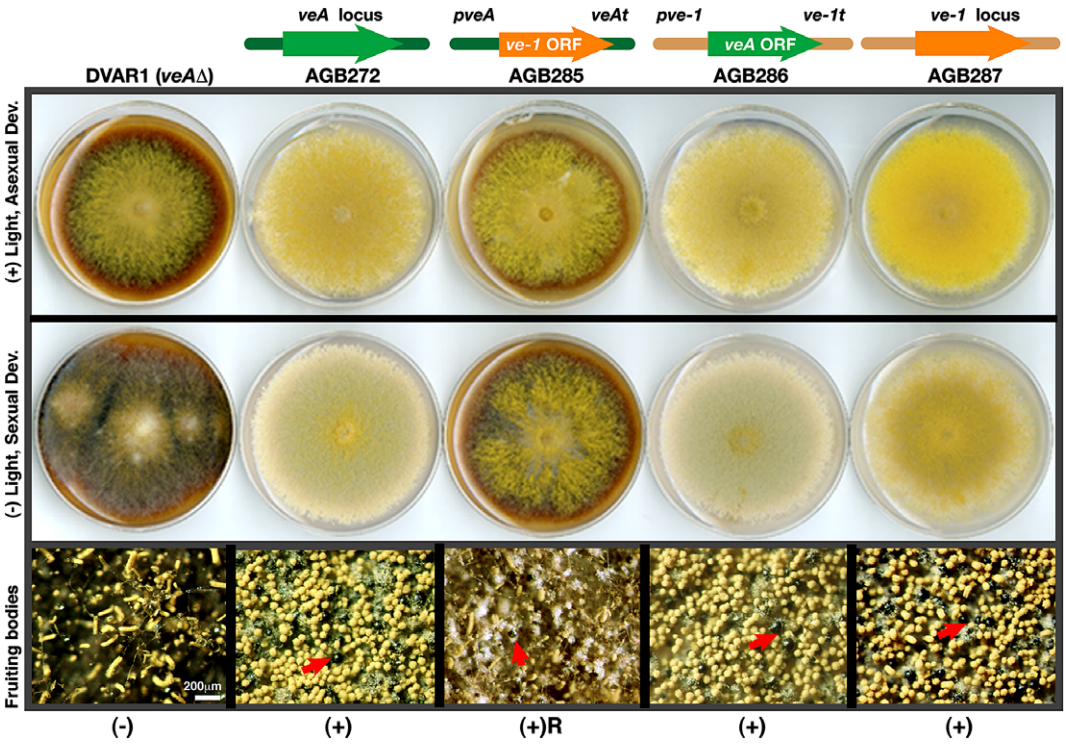

Fig. 4. The *N. crassa velvet* gene is a functional orthologue of *A. nidulans veA*. *A. nidulans* strain DVAR1 (*veAΔ*), which is impaired in fruiting body formation and defective in asexual sporulation associated with brown pigment production, was transformed with *velvet* genes from *A. nidulans* or *N. crassa*. As control, a native genomic fragment of *veA* was used that entirely rescued the *veA null* phenotype of DVAR1 (AGB272). Conidia (10^3) were inoculated per plate and incubated at 37 °C for 3 days in the presence of light for asexual development and for 5 days in the darkness to propagate sexual development, respectively. In strain AGB285, the *veA* deletion phenotype is partially complemented as brown pigmentation is reduced and the strain is able to form fruiting bodies with a delay of 4–5 days. pME3180 as expressed in AGB286 behaves identical to the native *A. nidulans* fragment. The *N. crassa ve-1* genomic fragment of 5 kb length fully complements all defects of DVAR1 (AGB287). The picture of the AGB285 fruiting bodies was taken on day 8 due to its delayed development, red arrows indicate mature cleistothecia.

After determining that *ve-1* expressed from the *veA* promoter is functional, we tested if *veA* expression in *A. nidulans* can be accomplished by the *ve-1* promoter. For that purpose, we devised a chimeric plasmid (pME3180) containing a *veA* cDNA under control of the *N. crassa ve-1* promoter and terminator sequences and transformed it into the *A. nidulans veAΔ* recipient DVAR1 to obtain strain AGB286. This construct fully rescued the *veAΔ* deletion phenotypes, which are characterized by the absence of fruiting bodies and excessive brown pigmentation (Fig. 4).

Finally, we tested whether a native genomic fragment of *ve-1* is also functional in *A. nidulans*. A PCR-amplified fragment of the *ve-1* genomic locus comprising 1.6 kb of 5′ and 3′ UTRs was transformed into the *veAΔ* strain DVAR1, yielding AGB287, and was found to fully restore the deletion phenotypes to result in ascospore-containing fruiting bodies and to reconstite the brownish pigmentation phenotype (Fig. 4). In all cases, the cleistothecia formed by any complementation construct were filled with fertile ascospores (data not shown).

We also quantified the number of conidiospores and fruiting bodies produced by the strains expressing the chimeric genes in comparison to the *veAΔ* progenitor DVAR1. Strains bearing the *veA* native genomic locus (AGB272) and the *ve-1* genomic locus (AGB287) produced comparable amounts of conidiospores, while in the strains that express chimeric constructs (AGB285 and AGB286), the defect in conidiospore production is only partially rescued (Fig. 5a). Furthermore, the amount of fruiting bodies generated in the chimeric *veA*-bearing strain (AGB286) is comparable to the *wild-type* situation of AGB272 (Fig. 5b), while cleistothecia formation in AGB287 (*ve-1* genomic fragment) is slightly lower. The low numbers of cleistothecia produced in AGB285 ($^{p}veA::ve\text{-}1::veA^{t}$) might be a result of interference of the 5′ intron that lies within the *veA* promoter with the intron in the *ve-1* coding sequence. Taken together, all three constructs complement the characteristic phenotypes of an *A. nidulans veAΔ* mutant strain, validating the functional conservation of the *velvet* factor between homothallic and heterothallic ascomycetous fungi. In *N. crassa*, the function of VE-1 in influencing asexual sporulation negatively is more obvious, but our data indicate a role of *veA* also in *A. nidulans* asexual development. Functionality of the *N. crassa ve-1* coding sequence and its promoter elements in *A. nidulans*, which also mirrors light-dependent regulation of expression, suggests that either the protein is regulated in the same manner or conserved promoter sequences are responsive to the light regulation in *A. nidulans*. The latter notion may be

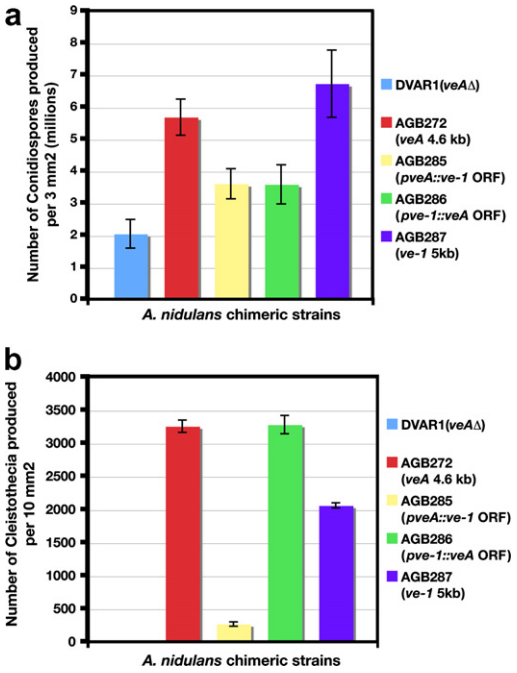

Fig. 5. Quantitative depiction of the cross-complementations studies on *N. crassa ve-1* constructs expressed in *A. nidulans*. (a) Asexual spore productions of chimeric strains per 3 mm^2: the DVAR1 strain produces reduced numbers of conidia, which is reconstituted by an *A. nidulans* genomic fragment comprising *veA* (AGB272) as well as by the chimeric constructs as expressed in strains AGB285, AGB286, and AGB287. (b) Cleistothecia produced per 10 mm^2: *A. nidulans veAΔ* strain (DVAR1) is defective in cleistothecia formation, and fruiting bodies' production is restored in strains AGB272 and AGB286. AGB287 also displays complementation of the *veAΔ* phenotype, however forming slightly less fruiting bodies than AGB272. Strain AGB285 forms less fruiting bodies with 4–5 days retardation. For each strain different sectors from three-independent plates were counted, standard deviations are indicated.

substantiated by the fact that the 5′ UTRs of both orthologous genes contain conserved regions.

4. Discussion

The recent sequencing efforts and publications on fungal genomes have revealed many orthologous and paralogous genes conserved throughout this kingdom. By detailed inspection of genes responsible for different metabolic, developmental, and morphological pathways functional or structural conservations could be uncovered. One interesting but also enigmatic fungal protein is the so-called *velvet* factor, which is unique for filamentous fungi. By analysis of the *N. crassa* genome, we disclosed one homologue to the *velvet*-encoding *A. nidulans veA* gene, and a sequence alignment of its deduced gene product VE-1 with VeA revealed a high degree of conservation. *A. nidulans* has an additional *velvet*-like protein designated as VelB, which has been identified as an interaction partner of VeA (our unpublished data); *in silico* analysis indicates that there is also a *velB* orthologue encoded by the *N. crassa* genome (NCU02775.3), suggesting conservation for this regulatory complex among ascomycetes.

Aspergillus nidulans VeA is required for the balance between asexual and sexual development (Kim et al., 2002). It is presumably inactivated by white or red light to stimulate sexual development in the darkness while it reduces asexual sporulation under these conditions (Champe et al., 1981; Mooney et al., 1990; Mooney and Yager, 1990; Timberlake, 1990). Red light regulation was elucidated partially by the characterization of an *A. nidulans fphAΔ* mutant that forms fruiting bodies inappropriately under red light illumination (Blumenstein et al., 2005). *N. crassa*, as a heterothallic ascomycete, displays two major life cycles: asexual conidiation and sexual development, and especially the latter is directed by internal and external factors. *N. crassa* fruiting bodies are produced independently of illumination, however, the perithecial ostiole is directed towards the source of light (Harding and Melles, 1983), and under blue light conditions, the number of protoperithecia is increased (Degli-Innocenti and Russo, 1984). In comparison, the *A. nidulans* illumination response is more pronounced with respect to red light, and there are no data describing any obvious UV or blue light response for this homothallic fungus.

Many genes that have been demonstrated to be involved in meiosis and ascus development are closely related in filamentous ascomycetes (Raju, 1992). In a sequence alignment, a striking conservation of the N-termini of the VeA and VE-1 proteins is evident (Fig. 1a), and there is functional evidence that this part of VeA is important for its regulatory function in *A. nidulans* development: many laboratory strains of *A. nidulans* carry a point mutation in the *veA* start codon which results in a truncated gene product (Kim et al., 2002). Strains expressing this *veA1* allele produce decreased numbers of fruiting bodies and are not affected in their sporulation by red light illumination (Mooney and Yager, 1990). To address any particular functional conservation of the N-termini, phenotypes resulting from expression of a correspondingly truncated *N. crassa* VE-1 protein might be assessed in future studies.

Our cross-complementation experiments demonstrate that VE-1, even when expressed from the heterologous *veA* promoter, is able to complement the phenotypes that derive from the loss of the *veA* gene in *A. nidulans.* The *ve-1* coding sequence under the control of the *A. nidulans veA* 5′ UTR rescued the *veAΔ* phenotype in a slightly delayed manner. Moreover, the *veA* coding sequence could be functionally expressed from the *ve-1* 5′ UTR, so the elements present in the promoter region of *ve-1* seem to be recognized by the transcriptional machinery of *A. nidulans* to result in proper expression of *veA*. Sequence alignments of the *ve-1* and *veA* 5′ UTRs provide evidence for conserved regions, which may serve as *cis*-acting elements. Although *ve-1* expression in *N. crassa* is induced upon red light illumination, the function of VE-1 is seemingly not influenced by environmental illumination conditions. *A. nidulans*' red light response is mediated by the phytochrome FphA (Blumenstein et al., 2005), which was found to interact with VeA (Purschwitz et al., 2006). The

N. crassa genome contains two phytochrome-like genes, that encode putative VE-1 interactors, and deletion of them results in no obvious phenotype (Froehlich et al., 2005). Deletion of *fphA* in *A. nidulans*, however, enhances fruiting body formation under red light illumination, which normally represses sexual development (Blumenstein et al., 2005). The presence of this red light-dependent phenotype in an *A. nidulans* but not an *N. crassa* phytochrome mutant might be an explanation why *ve-1* gene does not act in a light-dependent fashion in *N. crassa*, while it is apparently regulated in a light-dependent manner in *A. nidulans*. Accordingly, it is likely that VE-1 incorporates into the same signal transduction pathway in *A. nidulans*.

Deletion of *ve-1* in *N. crassa* caused the formation of shortened aerial hyphae accompanied by increased conidial production and a decreased tip extension with abnormal branching. One additional, more enigmatic phenotype is the lack of protoperithecia formation in SC medium, a phenotype that could not be complemented in any of the analysed knock-out mutant strains. Despite the fact that this sexual phenotype cannot be complemented by reintroduction of the otherwise complementing *ve-1* genomic fragment, it might retain some hints for an elusive role of VE-1 during the sexual development of *N. crassa*. Redundancy with other factors such as homologues of *velB* might obscure a function in protoperithecia formation. The pronounced aerial hypae phenotype seen in the *ve-1Δ* strain is similar to that observed in an *A. nidulans veAΔ* strain during sexual development in darkness. However, aerial mycelia formation of a *veAΔ* strain is not detectable during conidiation in the light. More interestingly, with the loss of *ve-1*, asexual sporulation is upregulated or derepressed in *N. crassa*, and the mutant forms more conidia than the *wild-type* strain FGSC987 (*74-OR23-1A*). This suggests that in *N. crassa ve-1* represses asexual sporulation either in the light or in the darkness, whereas *veA* represses asexual conidiation of *A. nidulans* in the darkness. In addition, it is also required for asexual sporulation in the light, since a *veAΔ* deletant is also defective in light-induced asexual conidiation (Kim et al., 2002). In *N. crassa*, *ve-1* overexpression from the *N. crassa cpc-1* promoter does not cause any abnormal phenotype (data not shown); in contrast, forced expression of *veA* in *A. nidulans* results in promiscuous production of sexual structures irrespectively of environmental conditions (Kim et al., 2002). Conclusively, *N. crassa* VELVET seems to be primarily a repressor of asexual conidiation, whereas *A. nidulans velvet* acts both as a repressor of asexual sporulation and as activator of fruiting body formation. Since the cross-complementation experiments confirm the functionality of VE-1 in *A. nidulans*, they likely operate similarly, and the observed difference could be due to different downstream elements. Taken together, VE-1 has retained some repressor function for asexual conidiation, but somehow lost its sexual role to another factor in *N. crassa*.

Despite of their structural conservation among filamentous ascomycete, *velvet* orthologues seem to have gained different roles depending on the organism's need, expanding their conserved role in developmental programs. They can be adapted to the fungal-specific life cycle and may be involved in diverse functions such as sclerotia formation and toxin production like in *A. parasiticus* (Calvo et al., 2004), nutrition-dependent sporulation like in *A. fumigatus* (Krappmann et al., 2005), or the microconidia-to-macroconidia ratio and cell wall formation like in the heterothallic fungus *Fusarium verticilloides* (Li et al., 2006). Understanding the mechanisms of such diverse *velvet*-driven roles among filamentous fungi will require more work at the level of protein–protein interactions, which holds the promise to elucidate the importance and exact regulatory mechanism of these structurally and functionally conserved fungal-specific proteins.

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