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The COP9 signalosome is an essential regulator of development in the filamentous fungus *Aspergillus nidulans*

Silke Busch, Sabine E. Eckert†, Sven Krappmann and Gerhard H. Braus*
Institut für Mikrobiologie und Genetik, Georg-August-Universität, Grisebachstrasse 8, D-37077 Göttingen, Germany.

Summary

The COP9 signalosome (CSN) is a conserved multiprotein complex involved in regulation of eukaryotic development. The deduced amino acid sequences of two *Aspergillus nidulans* genes, *csnD* and *csnE*, show high identities to the fourth and fifth CSN subunits of higher eukaryotes. The *csnD* transcript is abundant during vegetative growth as well as development and the corresponding protein accumulates in the nucleus. Strains deleted for either *csn* gene are viable and show identical mutant phenotypes at conditions that allow development: hyphae appear partly red and contain cells of reduced size. Additionally, light dependence of propagation onset is affected. The Δ*csn* mutants are capable of initiating the sexual cycle and develop primordia, but maturation to sexual fruit bodies is blocked. This developmental arrest could not be overcome by overexpression of the sexual activator velvet (VEA). We conclude that the COP9 signalosome in *A. nidulans* is a key regulator of sexual development, and its proposed structural and functional conservation to the CSN of higher eukaryotes enables studies on this regulatory complex in a genetically amenable organism.

Introduction

Development of multicellular organisms requires coordination of cellular processes, including cell cycle division, differentiation, intercellular communication and modifications of metabolic activities. One important regulatory mechanism that controls such complex processes in eukaryotes is targeted degradation of ubiquitinylated proteins by the 26S proteasome (Tyers and Jorgensen, 2000). Ubiquitinylation of the target proteins is mediated in an enzymatic cascade including E3 ubiquitin ligase complexes. The constitutive photomorphogenesis complex 9 (COP9), termed COP9 signalosome (CSN), directly interacts with E3 ubiquitin ligases (Schwechheimer *et al*., 2001; Suzuki *et al*., 2002). It is suggested to regulate the activity of E3 ligases towards its substrate proteins by deneddylation of the E3 cullin subunit (Lyapina *et al*., 2001; Zhou *et al*., 2001; Cope *et al*., 2002; Yang *et al*., 2002) and by phosphorylation of the target proteins (Bech-Otschir *et al*., 2001; Sun *et al*., 2002). The precise molecular mechanisms and cellular functions of the regulatory multiprotein complex CSN are the subject of current interest.

The eight subunits of the COP9 signalosome (Kapelari *et al*., 2000) are conserved in higher eukaryotes and characterized through PCI (proteasome, COP9, eIF3) or MPN (Mpr1p, Pad1 N-terminal) protein domains that are also part of two other multiprotein complexes, the lid of the 26S proteasome (LID) and the eukaryotic translation initiation factor 3 (eIF3) (Glickman *et al*., 1998; Kim *et al*., 2001). In mammalian cells, CSN is involved in several processes such as the control of hormone signalling and tumour growth by regulation of c-Jun and p53 protein levels (Li *et al*., 2000; Pollmann *et al*., 2001). In insects and plants, the COP9 signalosome is an essential regulator of development and its malfunction results in post-embryonic lethality (Wei *et al*., 1994; Freilich *et al*., 1999). Several subunits of the COP9 signalosome have been characterized in *Schizosaccharomyces pombe*, where this complex is not essential but fulfils deneddylation function (Mundt *et al*., 1999; 2002; Zhou *et al*., 2001). The complete genome sequence of *Saccharomyces cerevisiae* revealed only one putative though considerably less conserved homologue to CSN subunit 5 that associates with five additional proteins that exhibit deneddylation function, thus referred to as yeast COP9 signalosome (Wee *et al*., 2002).

The filamentous ascomycete *Aspergillus nidulans* is a model organism for the control of metabolic pathways

Accepted 6 May, 2003. *For correspondence. E-mail gbraus@gwdg.de; Tel. (+49) 551 39 3771; Fax (+49) 551 39 3820. †Present address: Department of Biosciences, University of Kent at Canterbury, Canterbury, Kent CT2 7NJ, UK.

and development in eukaryotes, because this fungus establishes highly specialized cell types and produces various secondary metabolites. After germination, hyphae require a minimum time of 16–20 h vegetative growth to achieve developmental competence and become susceptible to environmental signals that control induction of the asexual and sexual reproduction cycles (Axelrod *et al*., 1973). Knowledge about the regulatory system that drives the initial developmental decisions in *A. nidulans* is scarce. Prerequisite for development, with few exceptions, seems a medium–air interface (Axelrod *et al*., 1973). Light and aeration direct the differentiation towards the asexual cycle, whereas absence of light and increased partial pressure of carbon dioxide favour sexual propagation (Champe *et al*., 1994; Timberlake and Clutterbuck, 1994). The *veA* gene product is involved in light regulation and acts as a regulator that activates sexual development and represses asexual development (Kim *et al*., 2002).

The asexual reproductive unit of *A. nidulans* is the conidiophore, which forms green-pigmented conidiospores approximately 24 h after induction (Adams *et al*., 1998). The reproductive structure of the sexual cycle is the cleistothecium that harbours red-pigmented ascospores and requires approximately 80 h post-induction time for maturation (Braus *et al*., 2002): small hyphal aggregates ('nests') and globose Hülle cells with proposed nursing function surround the cleistothecial primordium. During further development, a rigid cleistothecial wall is built, embracing the meiotically derived ascospores. Because mutant strains defective in their sexual reproduction are still viable, this fungus is particularly suited for studies on sexual development. Genes involved in regulation of cleisothecia formation were mainly identified through *A. nidulans* mutants defective in distinct stages of sexual development: acleistothecial strains such as Δ*nsdD* and Δ*stuA* (Wu and Miller, 1997; Han *et al*., 2001), strains that stop the propagation programme after production of Hülle cells such as Δ*steA* or Δ*medA* (Clutterbuck, 1969; Vallim *et al*., 2000) and strains that are blocked in the maturation of immature cleistothecia (microcleistothecia) such as poorly supplemented amino acid auxotrophs or CPCA overproduction strains (Eckert *et al*., 2000; Hoffmann *et al*., 2000; Busch *et al*., 2001). Additionally, several mutant strains can build cleistothecial shells which are empty (Swart *et al*., 2001).

This work identified two components of the COP9 signalosome as a novel regulator of sexual development in *A. nidulans*. Deletion of the corresponding genes resulted in viable strains with multiple mutant phenotypes, most severe a block in maturation of primordia. This first description of the CSN in a filamentous fungus serves as a basis to study its function in an easily amenable, eukaryotic microorganism.

Results

The A. nidulans csnD *gene encodes a PCI domain protein similar to the fourth subunit of the COP9 signalosome*

Tagging mutagenesis of the *A. nidulans* wild-type strain R99 by restriction enzyme-mediated integration (REMI) (Schiestl and Petes, 1991; Sanchez *et al*., 1998) was used to screen for phenotypic defects in the formation of cleistothecia, the sexual fruit bodies of this fungus. Genetic analysis of the mutant strain AGB37 showed linkage between the single vector integration event conferring phleomycin resistance and the acleistothecial phenotype. The 560 bp genomic insert rescued from AGB37 served to isolate a genomic 9 kb *Xho*I fragment containing an open reading frame (ORF) of 1227 bp (Fig. 1A). Southern hybridization experiments showed a single copy locus of this gene in *A. nidulans* (data not shown). A corresponding cDNA was isolated via RT-PCR and comparison of both genomic and cDNA nucleotide sequences revealed that the coding region is not interrupted by an intron. The deduced peptide sequence of 408 amino acids with a calculated mass of 44.9 kDa contained a putative PCI domain (proteasome, COP9, eIF) and displayed identities of up to 38% to various proteins described as the fourth subunit of COP9 signalosome (CSN) (Fig. 2A). According to the signalosome subunit nomenclature (Deng *et al*., 2000) the gene was named *csnD*. Notably, identities were higher when CSND is compared with mammalia, vertebrates and plants than to the ascomycete *S. pombe*. The complete genome sequence of *S. cerevisiae* includes no orthologues for this subunit.

The csnD *gene is constitutively transcribed through the entire life cycle of* A. nidulans *and its gene product is enriched in the nucleus*

The COP9 signalosome is a regulator of development in *Drosophila melanogaster* and *Arabidopsis thaliana* (Wei *et al*., 1994; Freilich *et al*., 1999). We analysed the presence of *csnD* transcript in *A. nidulans* vegetative and differentiated cell material (Fig. 3A). Developmentally competent mycelia of a *csnD* wild-type strain (AGB162) were induced either asexually on unsealed plates or sexually on tape-sealed plates. At harvest, the asexual cultures showed immature and mature conidiophores respectively. Sexual cultures additionally differentiated Hülle cells, nests and mature cleistothecia. Northern experiments with RNA size standard revealed distinct *csnD* transcript signals of about 1.4 kb in length. Specific *csnD* mRNA overall signal intensities were comparably low but the ratios relative to the rRNA signals persist in the developing mycelia during all stages. As control, transcriptional levels of the metabolic housekeeping gene *gpdA*, encoding glyceraldehyde-3-phosphate dehydroge-

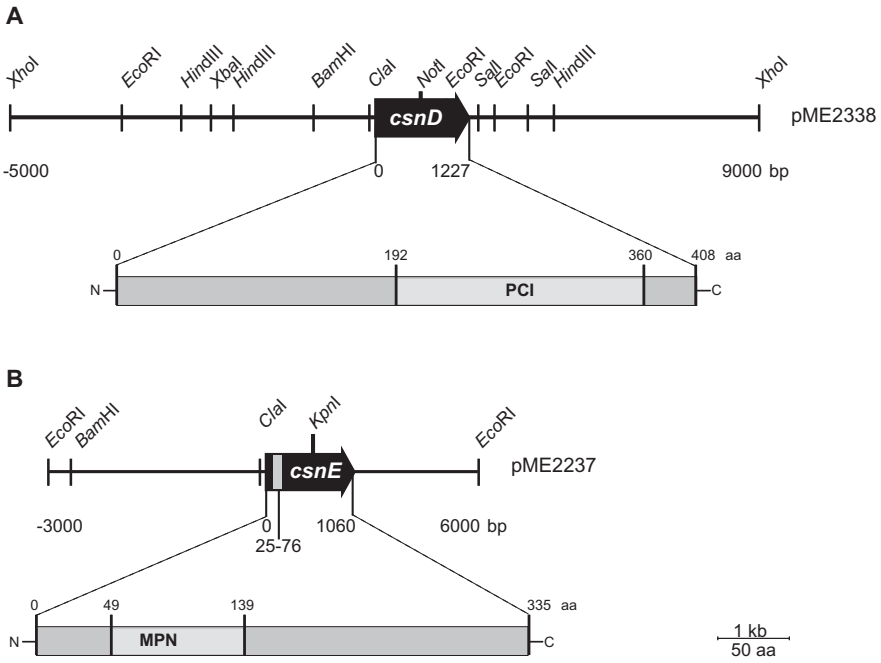

Fig. 1. The *Aspergillus nidulans* genes *csnD* and *csnE* encode PCI and MPN proteins typical for subunits of the COP9 signalosome. The genomic loci and the deduced proteins of *csnD* (A) and *csnE* (B) are shown. The intron of *csnE* is drawn as a grey box. Predicted PCI (proteasome, COP9, eIF3) and MPN (Mpr1p, Pad1 N-terminal) motifs are indicated.

nase (Punt *et al*., 1990), were monitored which significantly decreased as soon as development was induced.

Further attention was drawn to the localization of the CSND protein within the hyphal cell compartments by use of the green fluorescent protein (GFP) (Fernandez-Abalos *et al*., 1998). The complete *csnD* ORF driven by the strong inducible *alcA* promoter was fused to the *gfp* gene and ectopically integrated into wild-type strain AGB152. Under inductive conditions, the resulting P*alcA*::*csnD*::*gfp* strain (AGB197) grew and differentiated like the wild type (not shown) and the corresponding fusion protein was expressed as seen by fluorescence microscopy (Fig. 3B). In the majority of the hyphae, the fluorescence accumulated as distinct spots that co-localized with DAPI-stained nuclei. Such enrichment of CSN subunits in the nucleus, accompanied by a cytoplasmatic subcomplex of the CSN, was also observed in higher eukaryotes (Tomoda *et al*., 2002). This analogy in localization additionally accounts for the identity of CSND as a component of a COP9 signalosome in *A. nidulans*.

Deletion of the csnD *gene blocks sexual development and causes altered cell colour and size in* A. nidulans

An *A. nidulans* strain deleted for *csnD* was constructed to verify the acleistothecial phenotype of the REMI mutant strain AGB37. The complete ORF of *csnD* was replaced by a *pyr-4* expression cassette in the wild-type strain AGB152. Homologous integration of the *csnD* flanking regions into the genome was verified by Southern analysis (data not shown). After backcrossing of the transformants to the wild-type strain, the resulting deletion strain AGB195 produced no specific *csnD* transcripts as proven in Northern hybridization experiments. Radial colony expansion from a point spore inoculum on an agar surface resulted in similar growth rates with an average of about 0.37 mm radial vegetative colony growth per hour for the *csnD* deletion strain compared with 0.41 mm h^{-1} for the wild type at 37°C. In submerged liquid culture, where *A. nidulans* generally does not induce any development for lack of an air–medium interface (Axelrod *et al*., 1973), the *csnD* deletion strain was viable and did not show obvious anomalies of vegetative hyphae. However, when grown at an air–medium interface for a minimum of 48 h, two distinct peculiarities of hyphae became obvious: reduced cell size and red colouring (Fig. 4A). When the surface material of *A. nidulans* Δ*csnD* colonies was removed by washing the agar plates under the water tap, an aberrant reddish compact hyphal mat became visible that partly penetrated the agar surface. To facilitate microscopic analysis, cultures were grown on the surface of liquid medium which revealed the same hyphal traits. The *csnD* deletion strain produced, beneath apparently normal filaments, highly branched hyphae consisting of very short cells. Single hyphae showed a brownish-red pigmentation that was completely absent in mycelia from the wild-type strain. The red colouring appeared in a majority of the aberrant cells, but to a

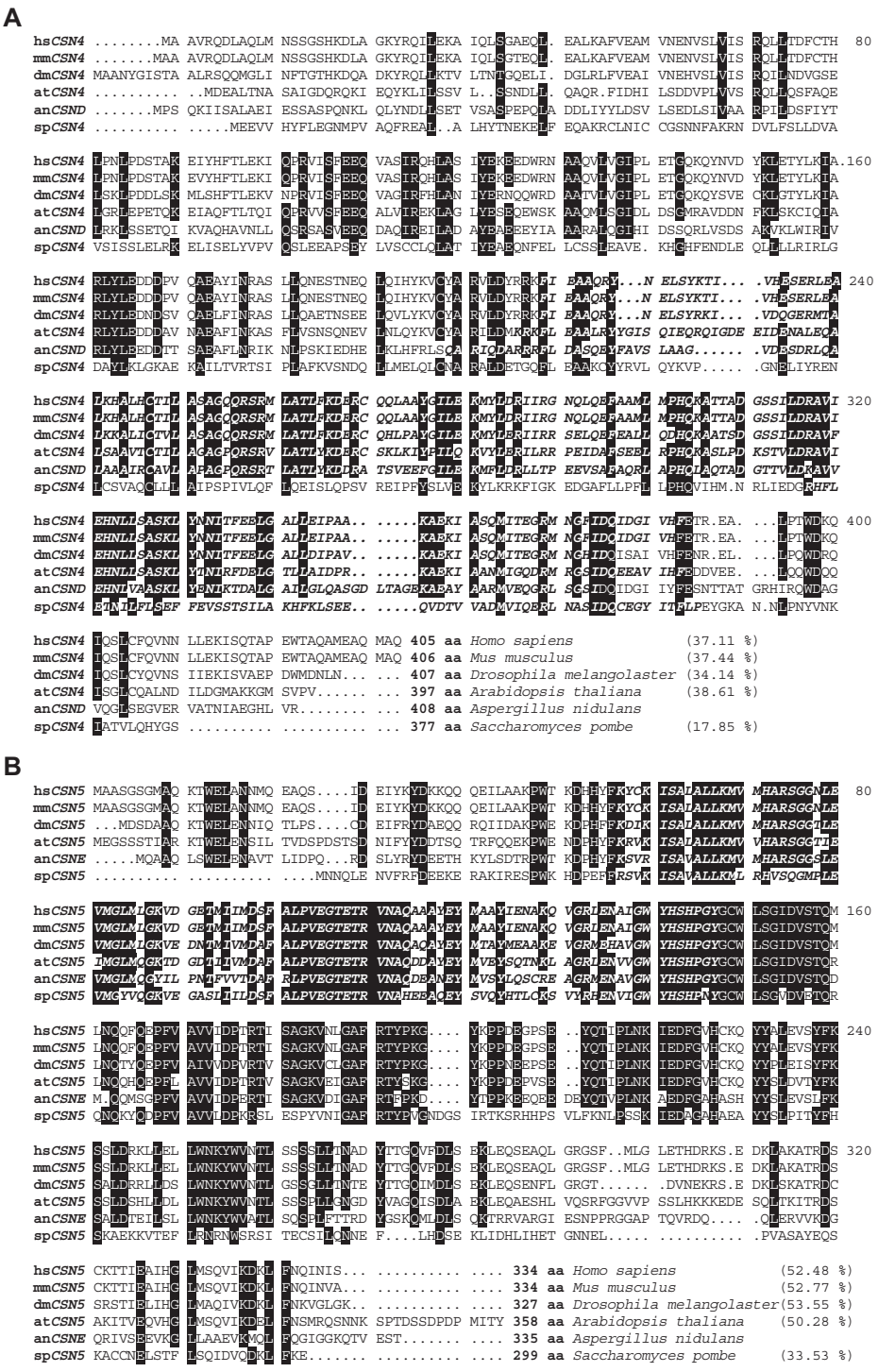

Fig. 2. CSND and CSNE show high amino acid identities to CSN4 and CSN5 of higher eukaryotes. The multialignments of deduced amino acid sequences of *csnD* (A) and *csnE* (B) to the corresponding sequences (Accession Number CSN4/CSN5) of *Homo sapiens* (NM016129/NP006828), *Mus musculus* (NM012001/NP038743), *Drosophila melanogaster* (AF129082/NP477442), *Arabidopsis thaliana* (AF176089/AAL58104) and *Schizosaccharomyces pombe* (NP593233/NP593131) is shown. Residues five times identical are highlighted. Predicted PCI and MPN domains are indicated by bold italic print and percentage of single sequence identities to the *A. nidulans* sequences is given in parentheses.

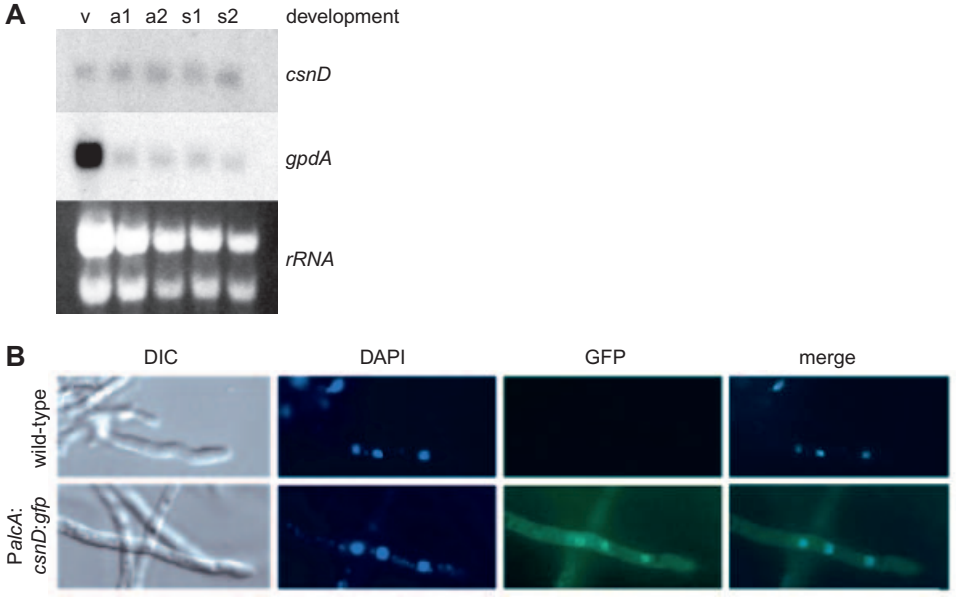

Fig. 3. *csnD* transcripts are abundant in vegetative and differentiated mycelia and a CSND:GFP fusion protein accumulates in the nucleus.
A. Northern experiments: mycelia of the wild-type strain AGB160 were synchronized by 18 h vegetative growth (v) in liquid culture and developmentally induced on solid medium to obtain asexual (a1: immature conidiophores; a2: mature conidia) and sexual (s1: nests, Hülle cells; s2: mature cleistothecia) tissue types for RNA isolation.
B. Wild-type strain AGB160 and strain AGB197 (P*alcA*::*csnD*::*gfp*) were grown overnight in liquid submerged culture under inductive conditions. Nuclei were stained with DAPI.

minor extent also in some morphologically wild-type like hyphae and conidiophore stalks. More careful observations of this phenomenon revealed that the centre of a *csnD* mutant colony discoloured earliest 48 h after inoculation. This colouring pattern spread towards the edge of the colony with time. We could not detect major secretion of a dye into the agar medium.

Conidiophore morphology of the *csnD* deletion strain (AGB195) resembled that of the wild type. Single colonies or a point inoculum of this mutant differentiated conidiospores predominantly, but not exclusively, in the centre of the colony. At confluent inoculation, but not in pre-grown developmentally competent mycelia (as described below), the number of conidiospores produced after 2 days was about the same range in both mutant and wild-type strains. Thus, the process of conidiation seemed not to be generally affected in the *csnD* deletion strain AGB195. By contrast, this mutant showed a severe defect in the sexual propagation cycle (Fig. 4A). The first morphologically visible stages of the sexual cycle, the nests accompanied by Hülle cells, harboured primordia which morphologically resembled that of the wild-type. But the next developmental step, formation of microcleistothecia, was never observed in the *csnD* deletion strain. Consequently, the mutant strain failed to produce mature cleistothecia with a rigid shell and red pigmented ascospores. This specific block in sexual development, together with the cell size and colouring defects, were complemented in strain AGB203 where a genomic wild-type *csnD* fragment was ectopically integrated in the *csnD* deletion mutant (Fig. 4B). Thus, all mutant phenotypes were dependent on the *csnD* gene.

An A. nidulans *strain lacking the fifth CSN subunit (CSNE) shows a* ΔcsnD*-like phenotype*

A second component of the proposed CSN was isolated and deleted to verify the existence of this complex in *A. nidulans*. A nucleotide stretch of 609 bp with similarities to CSN subunit 5 from *A. thaliana* was identified through sequence information obtained from the Monsanto Microbial Sequence Database and served to isolate a 6 kb genomic *Eco*RI fragment containing a coding region of 1060 bp (Fig. 1B). Southern hybridization experiments indicated that the coding region is present with one single copy in the genome (data not shown). The corresponding cDNA was isolated by RT-PCR and comparison with the genomic sequence revealed one intron of 51 bp. From the deduced peptide sequence of 335 amino acids a molecular mass of 37.8 kDa was calculated. The deduced amino acid sequence revealed a conserved MPN domain characteristic for the CSN subunit 5, and showed up to 53% identities to the fifth subunits of the COP9 complex from higher eukaryotes (Fig. 2B). The putative yeast CSN5 counterpart, Rri1p (Wee *et al*., 2002), exhibits only 20% identities (not shown). The gene was named *csnE*

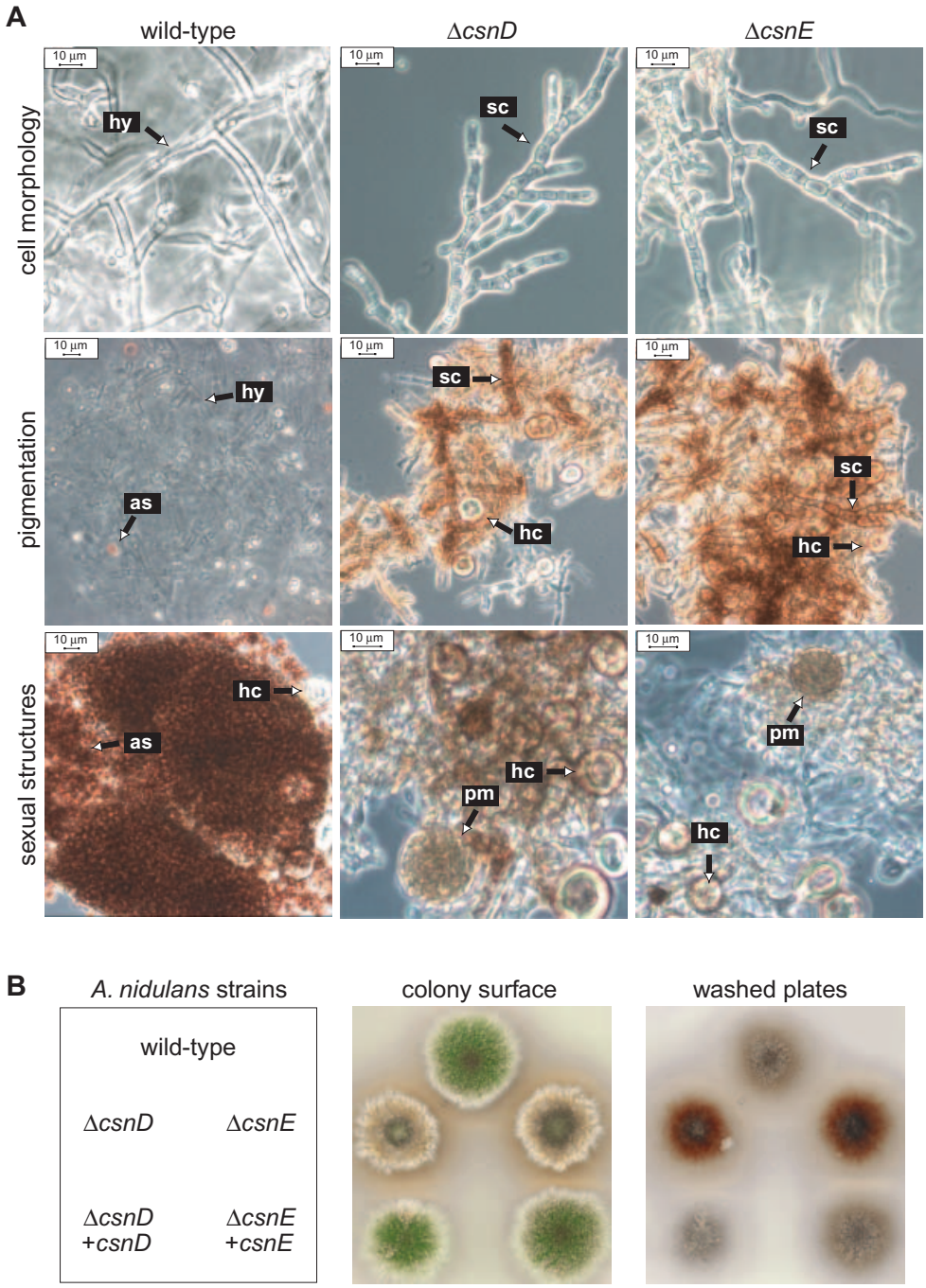

Fig. 4. *A. nidulans* strains that lack CSN subunits exhibit pleiotrophic mutant phenotypes. *A. nidulans* strains AGB195 (Δ*csnD*), AGB209 (Δ*csnE*) and wild-type strain AGB160 were grown on an air–medium interface (solid but for A row 1 liquid medium) to allow development.
A. Row 1: Both *csn* mutant strains produce wild-type-like vegetative hyphae (hy) and hyperbranched filaments consisting of aberrant short cells (sc). Row 2: Δ*csn* colonies contain red-coloured hyphae, including normal filaments and short cells. Row 3: Mature cleistothecia with ascospores (as) were produced by the wild type, whereas the *csn* mutant strains develop only Hülle cells (hc) and primordia (pr).
B. Ectopic integration of *csnD* (AGB203) and *csnE* (AGB211) into the *csn* deletion strains restored propagation phenotypes (left; conditions that allow both asexual and sexual development) and the aberrant red colouring of hyphae (right; surface material was removed by washing).

according to the unified COP9 signalosome nomenclature (Deng *et al*., 2000).

We substituted the 5′-terminal part of the *csnE* coding sequence with the *pyr-4* marker by homologous integration as proven in Southern experiments (data not shown). Similarly to the *csnD* deletion strain, the *csnE* mutant (AGB209) produced short cells and red hyphae when grown at a medium–air interface and ceased development

after formation of primordia (Fig. 4A). All mutant phenotypes were abolished in strain AGB211 where a genomic wild-type copy of *csnE* was ectopically integrated in the deletion strain (Fig. 4B). Thus, absence of either subunit 4 or subunit 5 of the COP9 signalosome in *A. nidulans* mediated identical pleiotrophic mutant phenotypes. This indicates that both, CSND and CSNE, are involved in the same function that controls several physiological and morphological processes.

Light dependence of development is disturbed in the A. nidulans csnD *deletion strain*

We questioned whether the regulatory role of the CSN in *A. nidulans* includes integration of environmental signals. In the plant *A. thaliana*, the COP9 signalosome was initially described as a regulator of light-induced development (Wei *et al*., 1994). Illumination is an important parameter for initiation of development in *A. nidulans*: light generally induces asexual and represses sexual development (Mooney and Yager, 1990). The only component of a proposed light-regulatory pathway in *A. nidulans* is the *veA* gene product, though the molecular function of this regulatory protein is still obscure. Overexpression of *veA* (OVAR5) mediates increased cleistothecial development whereas the *veA* deletion strain (DVAR1) is acleistothecial but conidiates (Kim *et al*., 2002). Additionally, DVAR1 produced a red hyphal colour reminiscent to the phenotype observed in the *csnD* deletion strains (Fig. 5A). Notably, most frequently used *A. nidulans* laboratory strains carry the *veA1* allele, a partial deletion of the ORF, which abolishes light dependence of conidiation and reduces production of cleistothecia and thus resembles a weak *veA* deletion phenotype (Mooney and Yager, 1990; Champe *et al*., 1994).

To analyse light dependence in *A. nidulans*, initiation of the two reproduction pathways was analysed in developmentally competent mycelia transferred to agar plates. The differential induction of the two reproductive cycles is generally not absolute, and for clarity, occurrence of occasional developmental structures (less that 25%) compared with the wild type was not taken into account. Wild-type strains predominantly induced the sexual cycle in the dark and the asexual cycle in the light (Fig. 5A). Changes in the *veA* locus abolish light dependence: *veA1* and Δ*veA* strains predominantly induced the asexual cycle whereas *veA* overexpression resulted in sexual induction. A Δ*csnD* strain predominantly induced the sexual cycle, independent of the light signal. Thus, deletion of *csnD* abolishes light dependence of development in *A. nidulans,* indicating that the COP9 signalosome of *A. nidulans* is essential for light-dependent signalling. The *csnD* deletion was combined with the *veA1* mutation of strain AGB162 and with the P*niiA*-driven *veA* overexpression of strain OVAR5. The resulting strains AGB192 and AGB220 both lacked light dependence of development, Δ*csnD*/P*niiA*:*veA* leads to induction of the sexual cycle, such as Δ*csnD* or P*niiA*:*veA* whereas Δ*csnD*/*veA1* leads to induction of the asexual cycle, such as *veA1*. Thus, with respect to light-dependent developmental induction, the *csnD* deletion did not change the *veA1* mutant phenotype.

The velvet gene product does not release the sexual block of csnD *deletion strains*

We investigated a molecular connection between *csnD* and *veA* in Northern hybridization experiments with RNA from developmentally competent mycelia of wild type, *csnD* deletion and *veA1* strains induced asexually or sexually. On the transcriptional level, specific *csnD* and *veA* mRNA signals, compared with rRNA, were not significantly altered after shift from vegetative to differentiating cultures in the wild-type strain and the *veA1* strain (Fig. 5B). *csnD* transcripts were present independent of the *veA* allelic state. Vice versa, the quantity of *veA* transcript was similar in *csnD* wild-type and deletion strains. Taken together, transcription of *csnD* and *veA* seem to proceed independently, irrespective of the developmental state of the culture.

Additionally, the genetic relationship between *csnD* and *veA* was analysed by means of mutant phenotypes. The Δ*csnD*/*veA1* strain (AGB192) showed the same red colour as well as aberrant cell sizes and also produced Hülle cells and primordia that never matured to cleistothecia, though apparently less than the corresponding *csnD*/*veA*+ strain (Fig. 5A). Thus, the *veA1* mutation is unable to suppress the block in development caused by *csnD* deletion. All phenotypes of the deletion mutant AGB192 were complemented by ectopic integration of the corresponding *csnD* genomic fragment in strain AGB193. The *A. nidulans* Δ*csnD*/P*niiA*:*veA* strain (AGB220) showed hyphae with red colouring and aberrant cells and a block of primordia maturation (Fig. 5A). This indicates that even overproduction of VEA, which normally leads to enhanced cleistothecia production, does not lead to development of mature cleistothecia in a *csnD* deletion strain.

Discussion

The COP9 signalosome is an essential regulator of development in higher eukaryotes with highly conserved functions. This study identifies the existence of the CSN in filamentous fungi and describes it as key regulator of fungal development. The *csnD* and *csnE* genes of *A. nidulans* encode the fourth and fifth CSN subunits including the characteristic PCI and MPN motifs (Kim *et al*., 2001). *csnD* mRNAs are present in both vegetative and developing cultures. Specific mRNAs for CSN subunits were also detected in all mouse embryonic and adult

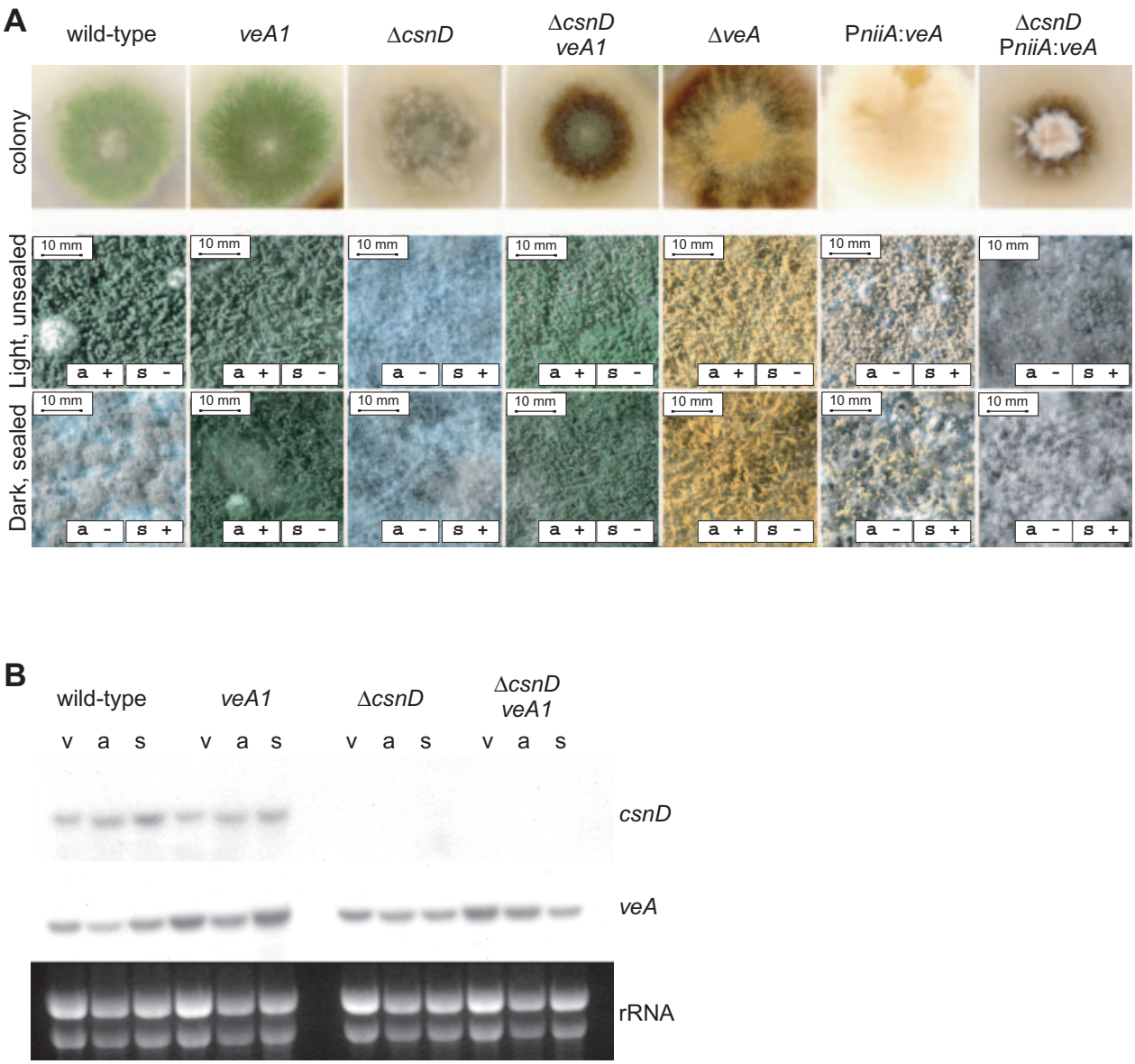

Fig. 5. CSN regulation affects light response but not *veA* transcriptional levels. *A. nidulans* strains with different *csnD* and *veA* allelic states were used: AGB160 (wild type), AGB195 (Δ*csnD*), AGB162 (*veA1*), DVAR1 (Δ*veA*), OVAR5 (P*niiA*::*veA*), AGB192 (Δ*csnD*; *veA1*) and AGB220 (Δ*csnD*; P*niiA*::*veA1*).
A. Top row: The overall colony phenotype on solid medium incubated in the light is shown. Lower rows: Light response of development was analysed with developmentally competent mycelia that were induced either asexually in the light (middle row) or sexually on sealed plates in the dark (lower row). Occurrence of any sexual tissue types was valued as induced sexual reproduction cycle. Induction (+) or repression (–) of asexual (a) or the sexual (s) reproduction is indicated.
B. Specific *csnD* and *veA* mRNAs are shown in Northern experiments of RNA from developmentally competent mycelia induced either asexually or sexually, resulting in vegetative (v), asexual (a) and sexual (s) tissue types.

tissue tested (Bounpheng *et al.*, 2000). A CSND::GFP fusion protein is dispersed in the cytoplasm and enriched in the nuclei. This is in agreement with observations in other organisms, where CSN subunits are predominantly localized in the nucleus as multiprotein complex, and subunits 4–8 were additionally found in the cytoplasm probably forming a smaller subcomplex (Kwok *et al.*, 1998; Tsuge *et al.*, 2001; Tomoda *et al.*, 2002). Therefore, the products of the two identified genes represent the first members of the COP9 signalosome in filamentous fungi.

In higher eukaryotes, defects in CSN function result in severe developmental phenotypes and post-embryonic lethality (Wei *et al.*, 1994; Freilich *et al.*, 1999), whereas malfunction of the complex in *S. pombe* is not lethal and leads to minor mutant phenotypes such as delayed progression through the cell cycle and increased sensitivity

to ultraviolet light (Mundt *et al*., 1999; Mundt *et al*., 2002). This work identified the COP9 signalosome of *A. nidulans* as a key regulator in the development of the organism, essential for regulation of colouring, cell size, light regulation and sexual reproduction. The study of the CSN in the model organism *A. nidulans* has major advantages: it is easily accessible to molecular manipulations, and in contrast to CSN defects in higher eukaryotes, an *A. nidulans* strain defective in its sexual cycle is viable and can propagate via its asexual cycle.

CSN in sexual development

The most severe mutant phenotype of *csnD* or *csnE* deletion in *A. nidulans* is a block in sexual development. To our knowledge, a specific developmental arrest at the level of cleistothecial primordia has not been described before. Initiation of the sexual cycle and differentiation processes leading to the general architecture of primordia are not impaired in the *csn* deletion strains, whereas further differentiation and maturation of wall and ascospores are blocked. Our work suggests that the COP9 signalosome is an essential player in the regulatory process that mediates maturation of primordia. A similar developmental block at a level of metamorphosis of a primordial to mature stage can also be observed in homozygous CSN mutants of the fruit fly *D. melanogaster*. The mutant embryos develop normally until the middle of the third instar, but then cease to develop and die (Freilich *et al*., 1999). More detailed analyses revealed defects in oocyte as well as embryonic development by misexpression of developmental factors (Oron *et al*., 2002). The block in sexual development seems to be the most severe phenotype of a defect in CSN function in *A. nidulans*. As stated above, the additional phenotypes of changed cell size and colour may also be related to developmental processes. It is thus conceivable that the COP9 signalosome in *A. nidulans* is dispensable for growth and housekeeping functions but essential for correct regulation of development.

CSN in vegetative growth

The COP9 signalosome of *A. nidulans* is involved in the control of the cell size which might hint at a connection to polar apical growth and lateral branching in surface-grown cultures. Generally, establishment of polar growth after germination and its later maintenance seem independent processes in *A. nidulans* (Momany *et al*., 1999). Young hyphae of surface-grown *csn* deletion colonies as well as hyphae grown in submerged liquid culture show no obvious aberrance. Thus, the establishment of polarity seems not to be generally disturbed in *csn* deletion strains, but maintenance of apical extension might be a target of CSN action. In wild-type strains, polarized apical growth seems to be turned off during developmental programmes. In the asexual cycle, polarized growth switches to a bud-like growth form during sterigmata formation and conidiation (Adams *et al*., 1998) and in the sexual cycle, branched filaments with knobby cells are described as ascogenous hyphae formed in immature cleistothecia (Braus *et al*., 2002). *A. nidulans* strains with overproduction of the transcription factor STEA block vegetative growth and produce highly branched hyphae with small, knobby cells very similar to ascogenous tissue, though a direct relation has not yet been proven (Vallim *et al*., 2000). Nevertheless, the short and highly branched hyphae in elderly *csn* deletion strains do not morphologically resemble the phenotype described for the STEA overproduction strain and young ascogenous tissue. Similar to the cell size defect of *A. nidulans csn* deletion strains, reduced function of the *A. thaliana* CSN causes increased secondary inflorescences and reduced internode length and cell size, primarily due to a loss of apical dominance. Apical dominance is driven by the phytohormone auxin which in turn is controlled by the CSN (Schwechheimer *et al*., 2001). Auxin seems also to play a role in development of *A. nidulans* (Eckert *et al*., 2000), thus future studies should focus on a possible coordination of development and hormone signalling by the *A. nidulans* CSN.

CSN in secondary metabolism

Malfunction of the CSN in *A. nidulans* leads to an aberrant red colour, possibly caused by a red pigment and thus connected to regulation of secondary metabolism. To date, we have no indication about the origin of this colouring matter. Wild-type strains deposit brownish melanin in walls of older hyphae when mycelia are grown in submerged liquid culture (Pirt and Rowley, 1969), a phenomenon not altered in the *csn* deletion mutants (not shown). It seems striking that the red colouring in the *csnD* mutant becomes visible after 2–3 days of growth which is the time scale where also the first structures of sexual development become visible. But to date, we have no indication that the red colour is related to asperthecin (Howard and Raistrick, 1955) which dyes the ascospores. The aberrant colouring of hyphae in the *csn* deletion strains appears independent of developmental induction by light and the allelic *veA* state. Regulation of the production of the putative red pigment thus seems to be mainly mediated by time and/or growth phase. Thus, an impact of the COP9 signalosome in the internal regulation of onset of secondary metabolism is conceivable. Astonishingly, red coloured hyphae are also seen in a *veA* deletion strain, but it is not clear whether the red pigment produced by Δ*csn* and Δ*veA* strains is identical.

CSN in light-dependent signalling

Its impact on colouring, cell size and sexual development suggests that the COP9 signalosome has several different downstream targets. This raises the question which upstream factors regulate CSN activity. An external signal important for development in *A. nidulans* is light, with the *veA* gene product as a proposed part of a corresponding signal transduction pathway. A *csnD* deletion strain is 'blind' to light regulation, such as strains with constitutively low or high *veA* expression. Thus, the COP9 signalosome of *A. nidulans* is involved in light-dependent signalling and may even be connected to the same signal transduction pathway as VEA. Notably, in the plant *A. thaliana*, the CSN is involved in the repression of photomorphogenesis in the dark by assisting in ubiquitinylation of a transcriptional activator of light-regulated genes (Osterlund *et al*., 2000; Suzuki *et al*., 2002). The product of the *A. nidulans veA* gene has a negative influence on initiation of the asexual but a positive on the onset of the sexual cycle (Kim *et al*., 2002). It seems striking that the *csnD* deletion strain in a velvet wild-type background acts like a *veA* overproduction strain: a constitutive induction of the sexual cycle. In analogy to the findings in *A. thaliana*, it is conceivable that the COP9 signalosome of *A. nidulans* mediates a negative post-transcriptional effect on VEA, resulting in increased VEA protein levels in a *csnD* deletion strain. The function of the CSN in light signalling might thus be genetically placed upstream or at the level of VEA, though this question should be addressed in future studies.

In summary, we present the first report of components of the COP9 signalosome in filamentous fungi and present strong evidence of its key regulatory function of development of the mould *A. nidulans*. The CSN of *A. nidulans* is involved in several cellular processes including colouring, cell size, light-dependent signalling and sexual development. The function of the COP9 signalosome in filamentous fungi resembles in some respects that of higher eukaryotes. This study represents an attractive basis to deliver new insights of the functions of the COP9 signalosome in eukaryotes.

Experimental procedures

Growth conditions, physiological and morphological studies

Strains of *A. nidulans* (Table 1) were cultivated at 37°C with minimal medium (Bennett and Lasure, 1991) supplemented as described previously (Käfer, 1977). Vegetative mycelia were obtained from submerged liquid culture and development was allowed by a medium–air interface. Asexual sporulation was induced by continuous white light and cleistothecia formation by oxygen-limiting conditions on tape-sealed plates in the dark (Clutterbuck, 1974). Development tests were performed with inoculated spores or with synchronized, developmentally competent mycelia that were pre-grown in submerged culture for 18 h. Radial growth tests were performed with about 500 conidiospores centred on an agar plate, with growth rates recorded as colony diameter within time. For conidiospore quantification (Bussink and Osmani, 1998), 4 ml of warm minimal medium containing 0.6% agar and about 1×10^6 spores was poured on corresponding solid medium. After 48 h, the top layer was

Table 1. *Aspergillus nidulans* strains.

Strain	Genotype	Reference: construction
A4	glasgow wild type	FGSC[a]
AGB10	*pyrG98*, *pyroA4*; *veA1*	Eckert *et al.* (2000)
AGB37	*nsd*::*bleo*	This work: R99 + pME1510
AGB152	*pyroA4*, *pyrG98*	This work: A4 × GR5
AGB160	*pyroA4*, *pyrG98*/*pyr-4*$^+$	This work: AGB152 + pRG3
AGB162	*pyroA4*, *pyrG98*/*pyr-4*$^+$ *veA1*	This work: AGB10 + pRG3
AGB192	*pyroA4*; *pyrG98*/*pyr-4*$^+$ Δ*csnD*; *veA1*	This work: AGB10 + pME2342
AGB193	*pyroA4*; *pyrG98*/*pyr-4*$^+$ Δ*csnD*/*csnD*::*bleo*; *veA1*	This work: AGB192 + pME2345
AGB195	*pyroA4*, *pyrG98*/*pyr-4*$^+$ Δ*csnD*	This work: AGB152 + pME2342
AGB197	*pyroA4*; *pyrG98*/*pyr-4*$^+$ P*alcA*::*csnD*::*gfp*	This work: AGB152 + pME2353
AGB203	*pyroA4*; *pyrG98*/*pyr-4*$^+$ Δ*csnD*/*csnD*::*bleo*	This work: AGB195 + pME2345
AGB209	*pyroA4*; *pyrG98*/*pyr-4*$^+$ Δ*csnE*	This work: AGB152 + pME2369
AGB211	*pyroA4*; *pyrG98*/*pyr-4*$^+$ Δ*csnE*/*csnE*::*bleo*	This work: AGB209 + pME2423
AGB220	*pyrG98*/*pyr-4*$^+$ Δ*csnD*; Δ*argB*::*trpC*ΔB/*argB*$^+$ P*niiA*::*veA*; *trpC801*	This work: AGB222 + pME2342
AGB221	*pyrG98*; Δ*argB*::*trpC*ΔB; *trpC801*	This work: AGB152 × DVAR1
AGB222	*pyrG98*; Δ*argB*::*trpC*ΔB/*argB*$^+$ P*niiA*::*veA*; *trpC801*	This work: AGB221 × OVAR5
DVAR1	*yA2*, *pabaA1*; Δ*argB*::*trpC*ΔB/*argB*$^+$ Δ*veA*; *trpC801*	Kim *et al.* (2002)
GR5	*pyrG98*, *pyroA4*; *veA1*; *wA3*	G. May[a]
OVAR5	*yA2*, *pabaA1*; Δ*argB*::*trpC*ΔB/*argB*$^+$ P*niiA*::*veA*; *trpC801*; *veA1*	Kim *et al.* (2002)
R99	arizona wild type	D. Geiser[a]
R99-6	*pabaA1*	D. Geiser[a]

a. Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City, KS, USA); Dave Geiser (Penn State University, University Park, PA, USA); Gregory May (University of Texas, Houston, TX, USA).

excised with the end of a 1 ml tip (about 1 cm^2), vortexed for 30 min in 0.5 ml of saline and spores were counted. Expression from the *alcA* promoter was induced by 2% ethanol or 10 mM cyclopentanone (Waring *et al*., 1989; Kennedy and Turner, 1996). Microscopy was performed by differential interference contrast (DIC) and fluorescence microscopy using a GFP filter set. Standard DAPI filter sets were used for visualization of nuclei stained with 4,6-diamidino-2-phenylindole (DAPI).

Molecular methods

Escherichia coli and *A. nidulans* transformations were performed as described (Inoue *et al*., 1990; Eckert *et al*., 2000). To obtain homogenized cell material, *A. nidulans* mycelia were filtered through miracloth (Calbiochem), frozen and ground in liquid nitrogen. RNA was isolated from 100 µl ground mycelia mixed with 1 ml of Trizol™ (Gibco BRL). Genomic DNA was isolated from a volume of 1 ml of mycelia (Lee and Taylor, 1990). Standard techniques were applied for Northern and Southern hybridization experiments (Southern, 1975; Rave *et al*., 1979). DNA for probes was amplified by PCR of plasmids pME2343 (*csnD*) with standard primers (T3/T7) and pME2352 (*veA*) with specific primers (5′-*Xho*I-TCA TTG AAT TGA ACA TCT TCG-3′/5′-*Hin*dIII-ATC GCG CCG GAT ACG GAC ACG-3′) or cut *Eco*RI/*Bam*HI from plasmid pAN5-22 (*gpdA*) (Punt *et al*., 1990).

REMI mutagenesis

REMI (Sanchez *et al*., 1998; Schiestl and Petes, 1991) of *A. nidulans* strain R99 was performed with 5 µg vector pME1510. For construction of pME1510, the PCR amplified multiple cloning site of pBluescript-II SK+ (Stratagene) was inserted into the blunt-ended *Eco*RI restriction site of pAN8-1 (Punt and van den Hondel, 1992) conferring phleomycin resistance. About 4200 phleomycin-resistant colonies (20 mg l^{-1} phleomycin in the medium) were generated with 5–20 U of different restriction endonucleases, with maximal transformation efficiencies of 150 transformants per µg vector. Fifty mutants with fluffy, acleistothecial or hyper-cleistothecial phenotypes were isolated. Single vector integration and linkage of the phenotype to phleomycin resistance was proven by Southern experiments and backcrossing to strain R99-6. For plasmid rescue, 10 µg restriction enzyme-cut genomic DNA was religated and transformed into *E. co*li SURE cells (Stratagene). Plasmid pME1661 containing a 560 bp insert of genomic DNA was rescued from REMI-mutant AGB37.

Isolation of genomic and cDNA

csnD and *csnE* were isolated by colony hybridization experiments of genomic *A. nidulans* sublibraries and subcloned into pBluescript-II KS+ (Stratagene), revealing plasmids pME2338 (*csnD*) and pME2237 (*csnE*). As probes, the REMI-rescued fragment of pME1661 was used for *csnD* whereas for *csnE* a PCR fragment was generated with a primer pair (5′-*Xba*I-GAG CTA GAG AAT GCT GTT ACC CTG-3′/5′-*Xba*I-CAA AGT CCT CGG CTT TGT TAA GCG-3′) deduced from database searches at the Monsanto Microbial Sequence Database for *A. nidulans* (http://microbial.cereon.com) and subcloned as pME2234. cDNAs were generated with RT-PCR with a polyT-primer and specific primers (5′-TAT GAA TCT AAT AGG TAC CAG AGA-3′/5′-CAG GAA GAG AAT ATA GTT ACG AGT-3′; 5′-TAT TCT CCA AAT CGA TAA TTA GC-3′/5′-CCA TCC AAA TAG ATC TAT ACG G-3′) and ligated via TA-cloning into pBluescript-II KS+ (Stratagene) resulting in plasmids pME2364 (*csnD*) and pME2363 (*csnE*) respectively.

Construction of plasmids for A. nidulans *manipulation (see* Table 2*)*

Deletion constructs contain the *pyr-4* expression cassette (*Eco*RI/*Ssp*I) of vector pRG3 (Waring *et al*., 1989): For deletion of *csnD*, the 3.5 kb *Eco*RI/*Hin*dIII fragment of pME2338 was subcloned into pBluescript-II KS+ (Stratagene). From the resulting vector pME1762 the flanking region was amplified via PCR (5′-CGC AGA CGG AAC AAC AGT-3′/5′-TTG AAT CAC AGC AGT GCA-3′) and blunt end ligated to the *pyr-4* marker as plasmid pME2342. For disruption of *csnE*, the 6 kb *Bam*HI/*Eco*RI fragment of pME2237 was subcloned into pUC19 (Vieira and Messing, 1982), the 5′ part of the coding region was removed via *Cla*I/*Kpn*I and substituted by the *pyr-4* marker via blunt end ligation resulting in vector pME2369. Complementation constructs were provided with the phleomycin resistance cassette of pME1510: the *csnD*-containing *Bam*HI/*Sal*I fragment of pME2338 was subcloned into pBluescript-II KS+ (Stratagene), opened with *Bam*HI/*Xba*I and ligated with the *Bgl*II/*Xba*I *ble* marker, resulting in plasmid pME2345. Accordingly, the *ble* marker was subcloned with *Xba*I/*Xho*I into pBluescript-II SK+, opened with *Xba*I and blunt end ligated with the *Eco*RI *csnE* fragment of pME2237 resulting in plasmid pME2423. For the GFP-fusion construct, the complete *csnD* ORF was PCR amplified from pME2338 (5′-*Kpn*I- ATG CCA TCC CAA AAG ATA ATC TCC-3′/5′-*Kpn*I-ACG TAC CAG ATG GCC CTC G-3′) was fused in frame to the *gfp* gene encoding GFP in plasmid pMCB32 (Fernandez-

Table 2. Plasmids constructed in this study.

Plasmid	Description	
pME1510	REMI vector	MCS; P*gpdA*:*ble*:T*trpC*
pME1565	Overexpression vector	*pyr-4*; P*alcA*-MCS-T*his2*
pME1661	*csnD* REMI-rescue of AGB37	500 bp genomic DNA
pME1762	*csnD* genomic	*Eco*RI/*Hin*dIII of pME2338
pME2234	*csnE* probe	609 bp *csnE* PCR-fragment
pME2237	*csnE* genomic	6 kb *Eco*RI fragment
pME2338	*csnD* genomic	10 kb *Xho*I fragment
pME2342	*csnD* deletion	P*csnD*:*pyr-4*:T*csnD*
pME2343	*csnD* probe	800 bp *csnD* fragment
pME2345	*csnD* complementation	P*gpdA*:*ble*:T*trpC*; *csnD*
pME2352	*veA* probe	500 bp *veA* PCR fragment
pME2353	*csnD* GFP fusion	P*alcA*::*csnD*::GFP
pME2363	*csnE* cDNA	1.2 kb
pME2364	*csnD* cDNA	1.6 kb
pME2369	*csnE* partial deletion	P*csnE*:*pyr-4*:T*csnE*
pME2423	*csnE* complementation	P*gpdA*:*ble*:T*trpC*; *csnE*

MCS, multiple cloning site; P, promoter; T, terminator.

Abalos *et al*., 1998) using the inserted *Kpn*I sites downstream of the *alcA* promoter resulting in plasmid pME2353.

A. nidulans *strain construction*

Strains used and constructed in this study are summarized in Table 1. Also, wild-type strains *A. nidulans* AGB152 and AGB10 served as *veA* and *veA1* genetic backgrounds respectively. Deletion of the *csn* genes was achieved by transformation of linearized deletion cassettes and selection for the *pyrG* auxotrophy marker. Homologous integration of pME2342 in *A. nidulans* strains AGB152 and AGB10 resulted in Δ*csnD* strains AGB195 and AGB192 respectively. Vector pME2369 deleted *csnE* in strain AGB152, resulting in strain AGB209. Both wild-type strains were transformed with vector pRG3 (Waring *et al*., 1989) resulting in control strains AGB160 and AGB162. All *csn* deletion strains were complemented by the *csn* wild-type genes of plasmids pME2345 and pME2423 via selection for phleomycin resistance, resulting in strains AGB203, AGB193 and AGB211 respectively. The *csnD* deletion was combined with the *veA* overexpression by transformation of plasmid pME2342 into strain AGB221, the latter was constructed by a series of crossing experiments between OVAR5, DVAR1, A4 and GR5 (Table 1). Ectopic integration of the *csnD*:*gfp* fusion plasmid pME2353 in strain AGB152 resulted in strain AGB197. All mutant strains were backcrossed to the genetic wild-type strain for purification. Homologous integration of the marker expression cassettes and ectopic integration of plasmids were verified by PCR and Southern hybridisation analyses.

Sequence analyses

DNA analysis was performed with the Lasergene software from DNASTAR. Pairwise alignments and the multiple sequence alignment with hierarchial clustering was performed using the software CLUSTALW (Corpet, 1988). Identification of protein motifs was performed *in silico* with PROSITE SCAN at http://hits.isb-sib.ch/cgi-bin/PFSCAN_parser. The nucleotide sequences of the *csnD and csnE* coding regions have been deposited in the GenBank database under GenBank Accession Numbers AF236662 and AY126455 respectively.

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