Fungal Genetics and Biology 56 (2013) 42–53

Contents lists available at SciVerse ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

conF and conJ contribute to conidia germination and stress response in the filamentous fungus Aspergillus nidulans

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article info

Article history:
Received 15 October 2012
Accepted 10 April 2013
Available online 1 May 2013

Keywords:
con-6
con-10
Conidia
Aspergillus nidulans
Light
Asexual development

abstract

Light induces various responses in fungi including formation of asexual and sexual reproductive structures. The formation of conidia in the filamentous fungus Aspergillus nidulans is regulated by red and blue light receptors. Expression of conidia associated con genes, which are widely spread in the fungal kingdom, increases upon exposure to light. We have characterized the light-inducible conF and conJ genes of A. nidulans which are homologs of con-6 and con-10 of Neurospora crassa. con genes are expressed during conidia formation in asexual development. Five minutes light exposure are sufficient to induce conF or conJ expression in vegetative mycelia. Similar to N. crassa there were no significant phenotypes of single con mutations. A double conF and conJ deletion resulted in significantly increased cellular amounts of glycerol or erythritol. This leads to a delayed germination phenotype combined with increased resistance against desiccation. These defects were rescued by complementation of the double mutant strain with either conF or conJ. This suggests that fungal con genes exhibit redundant functions in controlling conidia germination and adjusting cellular levels of substances which protect conidia against dryness.

1. Introduction

Numerous filamentous fungi live in soil, where it is dark and they encounter high humidity and little variation in temperature. When fungi reach the surface of the soil, they are exposed to UV radiation, desiccation or significant temperature changes, which require adaptation mechanisms to the harsh conditions on the surface (Rodriguez-Romero et al., 2012). The filamentous fungus Aspergillus nidulans reacts to light as environmental surface signal. Light promotes the formation of aerial hyphae with asexual spores (conidia) and reduces simultaneously the formation of sexual structures (cleistothecia). Within the soil where it is dark the fungus enhances sexual development and produces various secondary metabolites but delays asexual spore formation (Adams et al., 1998; Bayram and Braus, 2012; Bayram et al., 2010; Braus et al., 2002; Purschwitz et al., 2008). There are several studies that investigated various light receptors of A. nidulans, including the blue light receptors LreA and LreB (White Collar Complex, WCC) (Purschwitz et al., 2008), red light receptor phytochrome FphA (Blumenstein et al., 2005) and blue and UVA receptor cryptochrome (Bayram et al., 2008a). Light regulators physically and functionally interact with each other (Purschwitz et al., 2008) and the development is controlled by various light-dependent protein complexes (Bayram et al., 2008b; Purschwitz et al., 2008). Light control requires controlled protein degradation and a functional COP9 signalosome (Braus et al., 2011; Christmann et al., 2013; von Zeska Kress et al., 2012). The molecular mechanisms integrating the light signal are not yet fully understood and there are only limited studies on genes induced by light in this fungus. Microarray analyses revealed 425 light inducible genes in A. nidulans genome where a detailed characterization is missing (Ruger-Herreros et al., 2011).

In contrast to A. nidulans, there is much known about the light-dependent development mechanism of another model system Neurospora crassa (Ballario and Macino, 1997; Ballario et al., 1996; Corrochano, 2012; Corrochano et al., 1995; Froehlich et al., 2002; Linden and Macino, 1997). In initial studies with the ascomycete fungus N. crassa, several transcripts and polypeptides that are induced by light have been screened to elucidate the molecular mechanisms of light response, which are limited to carotenogenesis or morphogenesis (Chambers et al., 1985). con-6 and con-10 are expressed in vegetative mycelia upon blue light exposure, and this light regulation requires the white collar complex (WCC) (Corrochano et al., 1995; Lauter and Russo, 1991). The regulation of con-6 and con-10 by WCC and secondary photoreceptors, cryptochrome, opsin or phytochrome is complex (Olmedo et al., 2010). con-6 and con-10 are representatives of several conidiation genes (con gene) preferentially expressed during conidiation or in conidia (Berlin and Yanofsky, 1985; Roberts et al., 1988; Roberts and Yanofsky, 1989).

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1087-1845/$ - see front matter
http://dx.doi.org/10.1016/j.fgb.2013.04.008

CON-6 and CON-10 proteins are small proteins which are conserved among filamentous fungi, although deletions of these genes cause no obvious phenotype in N. crassa (Springer et al., 1992; White and Yanofsky, 1993). A. nidulans possesses two con-6 like isogenes conF and cffA and a single con-10 homologue, conJ (Fig. 1A). conJ as well as one of the two con-6 isogenes, conF, are induced by light (Ruger-Herreros et al., 2011). conF or conJ represent a significant number of approximately 300 genes that are preferentially expressed in conidia with unknown function (Timberlake, 1980; Zimmermann et al., 1980).

Most of the con genes are organized in several gene clusters on the A. nidulans genome (Galagan et al., 2005; Gwynne et al., 1984; Orr and Timberlake, 1982; Timberlake and Barnard, 1981). Twelve independent conidia-enriched transcripts (cet) were identified and assigned to four distinct classes (Osherov et al., 2002). cetD and cetJ code for small proteins which are similarly induced by light as conJ (Ruger-Herreros et al., 2011). CON-6 shows structural similarities to late embryogenesis abundant proteins (LEA) of maize (White and Yanofsky, 1993). LEA is defined as a subset of hydrophilins (Garay-Arroyo et al., 2000). Hydrophilins are thought to play roles in resistance to desiccation stress in many species (Battaglia et al., 2008).

In this study we have characterized A. nidulans con genes. We show that con genes exhibit redundant functions in controlling spore viability, germination and stress tolerance.

2. Materials and methods

2.1. Strains, media, and growth conditions

Strains used in this study are listed in Table 1. Standard laboratory Escherichia coli strains DH5α and MACH-1 (Invitrogen) were used for preparation of plasmid DNA. A. nidulans strains; AGB551 and AGB552 (Bayram et al., 2012) were used as wild type transformation hosts for the deletion and GFP tagging. A. nidulans strains were cultivated on minimal medium (MM) (0.52 g/l KCl, 0.52 g/l MgSO4, 1.52 g/l KH_2PO_4, 0.1% trace element solution, pH 6.5, (Barratt et al., 1965) at 37 °C and supplemented appropriately with 1 mg/l pyridoxine–HCl, 1 g/l uracil, 250 mg/l uridine or 1 mg/l PABA. For solid medium, 2% agar was added. For short-term light induction experiments, approximately 10^9 conidia were carefully inoculated on the surface of liquid media and cultivated in dark for 19 h at 37 °C, then cultures were exposed to white fluorescent light (90 lW m^{-2}) for 5, 10, 20, 40, 60, 120 min. As a control, cultures were further incubated in the dark for further 120 min. For developmental induction, the mycelia were grown vegetatively in order to synchronize the fungal cells and transferred to solid medium and grown for 2, 4, 6, 8, 12 and 24 h in light or dark at 37 °C. Total RNA was extracted from developmental and short-term light exposed liquid surface mycelia. For germination time course experiments with ConF, ConJ and GFP control strains was performed as follows: Freshly harvested conidia were inoculated into MM medium. Growing germlings and mycelia were harvested after 6, 12, 18, 20, 24 h growth. Proteins were extracted as given elsewhere (Bayram et al., 2012).

2.2. Extraction of RNA

For Northern experiments, mycelia were ground in liquid nitrogen and mixed with Trizol (Invitrogen) and chloroform. After centrifuging, the upper phase was extracted twice with phenol/chloroform (1:1 (v/v)). Total RNA was precipitated with isopropanol overnight, dissolved in cross-buffer and stored at −80 °C.

2.3. Hybridization techniques

Northern hybridization experiments were performed with digoxigenin (DIG) detection system (Roche) according to manufacturer's protocol. DIG labeled DNA probe of conF, conJ, cffA were amplified by PCR DIG probe synthesis kit (Roche) according to manufacturer's protocol.

2.4. Generation of linear ΔconF, ΔcffA and ΔconJ cassettes and construction of overexpression plasmids of each gene

Plasmids and oligonucleotides utilized and constructed in the course of this study are given in Tables 2 and 3, respectively. Oligos were purchased from (MWG-Biotech). All gene deletion experiments were carried out according to the method of (Takahashi et al., 2008) based on Latour system (Hirashima et al., 2006). To make conF deletion construct, 5′UTR region, 3′UTR region and ORF of conF were amplified from the wild type genomic DNA with primers SAT8/104, SAT105/106 and SAT107/34. The three amplicons were fused to the pyrG marker (from Aspergillus oryzae genomic DNA) with fusion PCR (nested oligos SAT12/108) yielding 4 kb linear deletion construct which was used to transform AGB551 into ΔconF intermediate strain (pyrG+). Conidia of the ΔconF strain (pyrG+) were inoculated on MM solid media with 5-FOA and uridine. 5-FOA resistant ΔconF strain (pyrG−) was selected (Table 1). To make cffA deletion construct, 5′UTR region, 3′UTR region and ORF of cffA were amplified from the wild type genomic DNA with primers SAT96/97, SAT98/99 and SAT100/101. These amplicons were fused to the A. oryzae pyrG marker with fusion PCR (nested oligos SAT102/103). The resulting 4 kb linear construct was used to transform into AGB551 to yield ΔcffA intermediate strain (pyrG+). 5-FOA resistant ΔcffA strain (pyrG−) was obtained as explained above. conJ deletion construct was made by amplifying 5′UTR (SAT14/109) and 3′UTR (SAT110/111) region and ORF of conJ (SAT112/44). Final 4 kb linear deletion construct containing A.o pyrG marker was used to obtain intermediate ΔconJ strain (pyrG+). ΔconJ strain (pyrG−) was obtained on 5-FOA media. cffA deletion cassette was transformed into ΔconF strain (pyrG−) to give intermediate ΔconF/ΔcffA strain (pyrG+). ΔconF/ΔcffA strain (pyrG−) was selected on 5-FOA media. conJ deletion construct was transformed into ΔconF strain (pyrG−) to yield intermediate ΔconF/ΔconJ strain (pyrG+) and ΔconF/ΔconJ double deletion (pyrG−). cffA deletion cassette was introduced into ΔconF/ΔconJ double deletion (pyrG−), which led to ΔconF/ΔconJ/ΔcffA strain (pyrG−) after 5-FOA selection.

2.5. Construction of C and N-terminally GFP-tagged conF and conJ plasmids

To create the C-terminal GFP-tag to conF and conJ, the entire ORFs with the promoter regions were amplified from the wild type genomic DNA with primers SAT30/31 or SAT37/38. SAT31 and 38 change the termination codon (TAA) of conF or conJ to GAA and add KpnI site. This KpnI site can ligate with the KpnI site of pJPE16, which fuses conF or conJ gene with egfp gene in-frame on the vector. pJPE16 carries long version of pabaA marker (5.4 kb) which enables reconstitution of an intact copy of the pabaA gene by homologous integration of the circular plasmid at pabaA locus in AGB552 strain. Integration resulted in duplicated pabaA (one is functional and the other is null) sequences flanking plasmid sequences. In order to construct N-gfp::conF fusion under native promoter, conF 5′UTR (OSBS11/13), conF ORF (OSBS14/15), conF 3′UTR (OSBS16/17) were amplified from the wild type genomic DNA. 5′UTR, gfp, conF ORF, pyroA, 3′UTR fragments were cloned in SmaI site of pUC19 by using in-Fusion cloning kit (Clontech) pOB325. Similarly, conJ 5′UTR (OSBS19/21), gfp, conJ ORF (OSBS22/23), pyroA, conJ 3′UTR (OSBS24/25) were joined and cloned (pOB326).

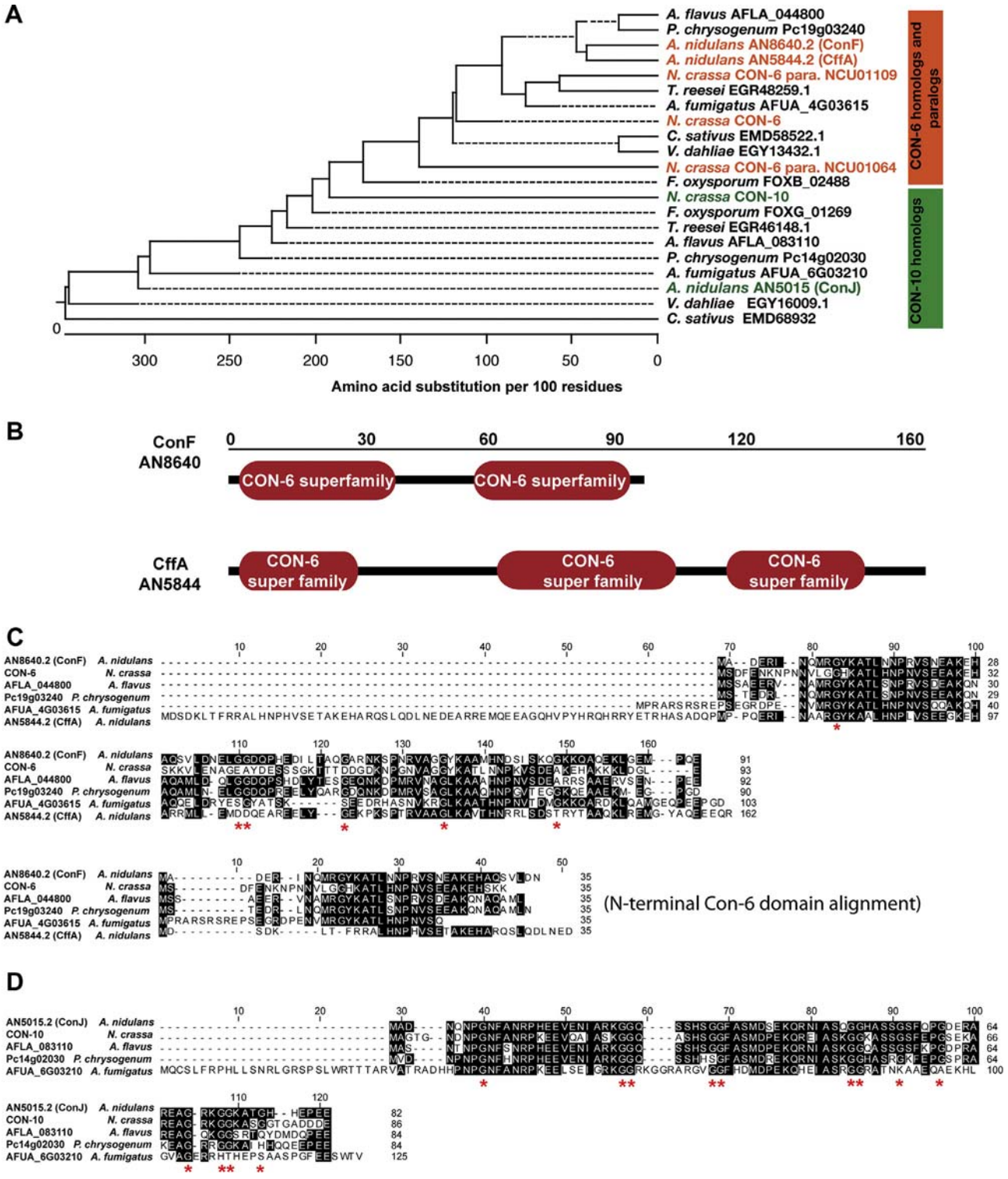

Fig. 1. Phylogenetic tree and domains found in Con proteins. (A) The phylogenetic tree of Con homologous proteins. The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987). Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Neurospora crassa CON-6 and CON-10 proteins were used as reference. Following CON-6 and CON-10 homologs and paralogs were applied in phylogenetic tree. CON-6 homologs: Aspergillus flavus (AFLA_044800), Penicillium chrysogenum (Pc19g03240), Aspergillus nidulans (ConF, AN8640.2), (CffA, AN5844.2), N. crassa CON-6 paralog 1 (NCU01109), paralog 2 (NCU01064), Trichoderma reesei (EGR48259.1), Aspergillus fumigatus (AFUA_4G03615), Cochliobolus sativus (EMD58522.1), Verticillium dahliae (EGY13432.1). Fusarium oxysporum (FOXB_02488). CON-10 homologs: N. crassa CON-10, F. oxysporum (FOXG_01269), T. reesei (EGR46148.1), A. flavus (AFLA_083110), P. chrysogenum (Pc14g02030), A. fumigatus (AFUA_6G03210), A. nidulans (AN5015, ConJ), V. dahliae (EGY16009.1), C. sativus (EMD68932). (B) Conserved domains of CON-6 superfamily in ConF and CffA proteins. (C) Global and N-terminal alignments of CON-6 homologs from different fungal species. D. Global alignment of CON-10 homologs shows Glycine residues. Glycine content of ConF (AN8640) and ConJ (AN5015) proteins is more than 8%.

Table 1
Strains used in this study.

Strain	Genotype	Reference
A. nidulans		
AGB551	nkuAD::argB, pyrG89, pyroA4, veA+	Bayram et al. (2012)
AGB552	nkuAD::argB, pabaA1, veA+	Bayram et al. (2012)
DconF	conFD, nkuAD::argB, pyrG89, pyroA4, veA+	This study
DcffA	cffAD, nkuAD::argB, pyrG89, pyroA4, veA+	This study
DconJ	conJD, nkuAD::argB, pyrG89, pyroA4, veA+	This study
DconF/DcffA	conFD, cffAD, nkuAD::argB, pyrG89, pyroA4, veA+	This study
DconF/DconJ	conFD, conJD, nkuAD::argB, pyrG89, pyroA4, veA+	This study
Dcon triple	conFD, cffAD, conJD, nkuAD::argB, pyrG89, pyroA4, veA+	This study
conF GFP	conF::egfp-pabaA; nkuAD::argB, pabaA1, veA+	This study
conJ GFP	conJ::egfp-pabaA; nkuAD::argB, pabaA1, veA+	This study
N GFP conF	N-gfp::conF, AfpyroA, nkuAD::argB, pyroA4, pyrG89, veA+	This study
N GFP conJ	N-gfp::conJ, AfpyroA, nkuAD::argB, pyroA4, pyrG89, veA+	This study
conF complementation	5′ UTR conF-pyroA 3′ UTR DconF/DconJ	This study
conJ complementation	5′ UTR conJ-pyroA 3′ UTR DconF/DconJ	This study
E. coli		
MACH-1	F- u80(lacZ)DM15 DlacX74 hsdR(rK− mK+) DrecA1398 endA1 tonA	Invitrogen
DH5a	F′ U80dlacZDM15, D(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(r_K^- ; m_K^+), phoA, supE44, k− thi-1, gyrA96, relA1	Hanahan (1983)

Table 2
Plasmids employed in this study.

Plasmid	Description	Reference
pJET1.2	Cloning plasmid	Fermentas
pUC19	Cloning plasmid	Fermentas
pOB325	N-gfp::conF-pyroA in SmaI site of pUC19	This study
pOB326	N-gfp::conJ-pyroA in SmaI site of pUC19	This study
pOB327	5′ UTR conF-pyroA 3′ UTR in SmaI site of pUC19	This study
pOB328	5′ UTR conJ-pyroA 3′ UTR in SmaI site of pUC19	This study
pJPE16	egfp::trpC-pabA in pJET1.2	This study
pJPEconF	conF::egfp::trpC-pabA in pJET1.2	This study
pJPEconJ	conJ::egfp::trpC-pabA in pJET1.2	This study

2.6. Construction of conF and conJ complementation plasmids

conF complementation construct was created by amplifying 5′UTR and conF ORF (OSBS11/15), conF 3′UTR (OSBS16/18), which were fused to pyroA by PCR (OSBS11/18) and cloned into SmaI site of pUC19 (pOB327). pOB327 served as a template for amplification of complementation cassette with primers OSBS12/17. Likewise, 5′UTR and conJ ORF (OSBS19/23), conj 3′UTR (OSBS24/26) were fused to pyroA and subsequently cloned in pUC19, which resulted pOB328. ConF complementation fragment was amplified from pOB328 with OSBS20/25.

2.7. Transformations

Transformation of E. coli and A. nidulans was performed as described (Hanahan et al., 1991; Punt and van den Hondel, 1992).

2.8. Immunoblotting

For detection of ConF and ConJ GFP fusions, 40 lg protein extract was used from germinating or light induced cultures. Western blot experiments were performed as described elsewhere (Christmann et al., 2013; Sarikaya Bayram et al., 2010).

2.9. Sequence analyses

The amino acid sequence of ConF, CffA and ConJ from A. nidulans were retrieved from AspGD "Aspergillus Genome Database" http://www.aspergillusgenome.org/. Reference sequences were retrieved from the National Center for Biotechnology Information Entrez Protein database. Multiple sequence alignments were carried out using ClustalW implement in MEGA5 (Tamura et al., 2011) with default parameters and drawn by GENETYX ver. 6. The phylogenetic trees were constructed using the Neighbor-Joining method (Saitou and Nei, 1987) by using MEGA5 (Tamura et al., 2011).

2.10. Desiccation stress tolerance test

Desiccation stress tolerance tests were carried out as follows. Conidia suspensions were prepared as described (Sarikaya Bayram et al., 2010). Two-day old conidia (10^5 per plate) of wild type and the mutants were spread on solid minimal medium (MM) with appropriate supplements and incubated at 37 °C. After 2 days the conidia were collected and counted by particle counter analyzer (MULTISIZER, BECKMAN COULTER). 10 ll spore solution containing 10^4 two-day old conidia of wild type and the mutants in test tube with rid open were dried completely at room temperature 30 min. by centrifugal vaporizer. Dried conidia were incubated at 37 °C for 0, 1, 3 and 5 days. Then conidia were rehydrated by 1 ml of water and approximately 200 conidia were immediately inoculated on solid MM and incubated for 2 days at 37 °C. Survival rates were calculated as a ratio of the number of growing colonies to the number of spores inoculated. These tests were performed in triplicate.

2.11. Germination rate calculation

Germination of wild type and transformants were monitored hourly in minimal medium supplemented as appropriate at 37 °C. Germlings were counted under light microscope. These tests were performed in six replicates.

2.12. Polyols and trehalose measurement

Soluble sugars and polyols for HPLC analysis were extracted from conidia. 2×10^8 two-day old conidia of the wild type, double, triple disruptant and complementation strains were suspended in 300 ll of H_2O and incubated at 98 °C for 3 h. Then the suspension were put in Microcon Centrifugal Filter Devices (Millipore), centrifuged for 20 min. at 23,000g and flow through containing polyols and sugars were collected. Before use, Microcon cartridges were washed by total 3 l H_2O for two overnight with stirring to remove glycerol on the membrane and confirmed that there were no residual glycerol by HPLC. In our experimental conditions the retention time of glycerol, erythritol, arabitol, mannitol and trehalose were 4.40, 5.08, 5.80, 6.75 and 10.82 min., respectively, upon chromatography onto a Shodex Asahipak NH2P-50 4E

Table 3
Oligonucleotides used in this study.

Designation	Sequence in 5′ → 3′ order	Features
SAT8	CGT GCT CAG TTT TGC GGC CTC	conF deletion 5′ outer
SAT12	CGT TAT ACT CTT GCC GGG CTC G	conF 5′ flanking 5′ inner
SAT14	CGA CGA GGG TGC CTC TAT GAG	conJ deletion 5′ flanking outer primer
SAT18	AGC GAC TCT TTC CAG CTT CCT CC	conJ deletion 5′ flanking innner
SAT30	CAG GTA CCT TCT TCC TGC GGC ATC TCG CC	conF ORF 3′ end T/G KpnI
SAT31	CAC CTG CAG GCT AGA CAG GTT AAC ATT TCT GCT C	conF promoter 963 5′r
SAT34	CAC CTG CAG GAT TAT TCT TCA CCA TTA TCG TAT CTG	conF deletion 3′ outer
SAT37	CAG GTA CCT TCT TCC TCA GGC TCG TGG TGA C	conJ ORF 3′ end T/G KpnI
SAT38	CAC CTG CAG GCT CGC ATT CCT CAC TTG ACA TC	conJ promoter 1500 5′
SAT44	CAC CTG CAG GGA CAG GTC ATA GAT CCA GTC TGT GG	conJ deletion 3′ outer
SAT45	CAC CTG CAG GCC TCA CTC GTC ATC ATC GCA GAC	conJ ORF 3′ inner
SAT46	AAG AGG TGG AAT TTA TCT GGC CTT G	A. oryzae pyrG 5′
SAT47	CTT TGG TCT CTA CGA GAG CAC C	A. oryzae pyrG 3′
SAT96	GGT TCG CAC AAA CTC CCA GTA TG	cffA deletion 5′ outer
SAT97	GTC TCC TAT CCC GTT TTC GTC CTT AGG ACT TGC	cffA deletion 5′ flanking 3′ primer with 5′ end of 3′ flanking
SAT98	ACG AAA ACG GGA TAG GAG ACG AAG AAG GAG AAG G	cffA deletion 3′ flanking 5′ primer with 3′ end of 5′ flanking
SAT99	CCA GAT AAA TTC CAC CTC TTG CAT GAA GTC CGC TTA ACT GTC	cffA deletion 3′ flanking 3′ primer with pyrG 5′
SAT100	TGC TCT CGT AGA GAC CAA AGA TGG ATT CTG ACA AGC TGA CCT TC	cffA deletion ORF 5′ primer with pyrG 3′
SAT101	CTA TCT CTG CTC TTC TTC CTG GG	cffA deletion ORF 3′ outer primer
SAT 102	CAG TGA GGG CAA AGC CGG AC	cffA deletion 5′ flanking inner primer
SAT103	TCT CGC GCA GTT TCT GAG CG	cffA deletion ORF 3′ inner primer
SAT104	TGT CAG GGA CGA AAT TGG GCG AGA TGC CGC AG	conF deletion 5′ flanking 3′ primer with 5′ end of 3′ flanking
SAT105	GCC CAA TTT CGT CCC TGA CAT CAA TCA GTC TGC	conF deletion 3′ flanking 5′ primer with 3′ end of 5′ flanking
SAT106	CCA GAT AAA TTC CAC CTC TTT GGA CTC GTA CTT GCC GTC CA	conF deletion 3′ flanking 3′ primer with pyrG 5′
SAT107	TGCTCTCGTAGAGACCAAAGTCTTGCGCCTGCTTTTTCCCCT	conF deletion ORF 5′ primer with pyrG 3′
SAT108	GTC AAC GTG AGC GAT GTT CTC GG	conF deletion ORF 3′ inner primer
SAT109	GTT CGC CTG GCC AAA CCC CGT GGC ATA CGT AC	conJ deletion 5′ flanking 3′ primer with 5′ end of 3′ flanking
SAT110	CGG GGT TTG GCC AGG CGA ACG GCA AAG AGA TTC	conJ deletion 3′ flanking 5′ primer with 3′ end of 5′ flanking
SAT111	CCA GAT AAA TTC CAC CTC TTC TAG AAT CGA TCA TCG GCT GCG	conJ deletion 3′ flanking 3′ primer with pyrG 5′
SAT112	TGC TCT CGT AGA GAC CAA AGG AAA CTT TTG GCC CTT TTT AGC ATG CC	conJ deletion ORF 5′ primer with pyrG 3′
OSBS11	TCG AGC TCG GTA CCC CTGT GTG CCA GCG CTT CAT C	conF 5UTR pUC19 fusioner
OSBS12	CTG TGT GCC AGC GCT TCA TC	conF 5UTR nest
OSBS13	CGC CCT TGC TCA CCA TGT AAT GTA CTA AAA AGT GGT GCT	conF 5UTR N-GFP fusioner
OSBS15	CCA GCA TCT GAT GTC CGA ATA TGC TCG TGA AAC ATT ATT CAG	N-GFP-conF fusioner
OSBS16	GCC TCC TCT CAG ACA GTG CAT CTG TTA CTA ATC CTC G	conF-pyroA fusioner
OSBS17	CCC TCA ATA GTT TCG TAT CAT AC	conF 3UTR nest
OSBS18	TCT AGA GGA TCC CCC CCT CAA TAG TTT CGT ATC ATA C	conF 3UTR pUC19 fusioner
OSBS19	TCG AGC TCG GTA CCC GCA TCT GGT GAC GAG CAT AGC	conJ5UTR pUC19 fusioner
OSBS20	GCA TCT GGT GAC GAG CAT AGC	conJ5UTR nest
OSBS21	CGC CCT TGC TCA CCA TGA TGT ATT TAA AG AAT TGG TTG TGG	conJ5UTR N-GFP fusioner
OSBS22	GGG TGG TAG CGG TGG TAT GGC CGA CAA CCA GAA CCC	N-GFP-conJ fusioner
OSBS23	CCA GCA TCT GAT GTC CCC CAA TCG TCA GAT CGT ATC	conJ-pyroA fusioner
OSBS24	GCC TCC TCT CAG ACA GCC ATG CAC TTC CAC TCA TGT AC	conJ3UTR nest
OSBS25	CCA CAG GAA TCA ATA CAA CCG	conJ5UTR pUC19 fusioner
OSBS26	TCT AGA GGA TCC CCC CAC AGG AAT CAA TAC AAC CG	conJ5UTR nest

(Shodex) using an isocratic elution with CH_3CN/H_2O (75/25) at 1.0 ml ml^{-1} detected by differential refractive index detector. Quantification of polyols and trehalose were performed by internal standard method on HPLC. 0.5 mg/ml of glucose as an internal standard was added to each samples and values of polyols and trehalose contents were obtained using the calibration curves of standard samples. Conidia of each strain was harvested from six independent cultures.

2.13. Confocal spinning disk microscopy

Freshly harvested A. nidulans spores (1000) were either inoculated on an agar surface on the cover glass (for asexual spore microscopy) or in eight chambered coverglass system (Nunc) supplemented with minimal medium. Asexually-induced cells were grown for 24 h, and vegetative cells were allowed to germinate for 10 h at 37 °C. Confocal images were taken with a Quantem :512SC (Photometrics) camera connected through a scanner unit (Yokogawa) to an Axiovert Observer.Z1 (Zeiss). During capturing and processing of the images the Slidebook v 5.0.1 (Intelligent Imaging Innovations) was used. The same exposure time (800 ms) was used to capture the images of the fluorescently labeled fungal strains during germling formation. Conidia fluorescence was observed in 200 ms setting.

3. Results

3.1. con-6 and con-10 are highly conserved among filamentous fungi

The A. nidulans genome contains two con-6 homologs (conF:AN8640, cffA:AN5844) and one con-10 homolog (conJ:AN5015) (Fig. 1A). Multiple global alignments with various CON-6 or CON-10 homologs and paralogs assigned ConF and CffA to the CON-6 group and ConJ to the CON-10 like proteins.

The open reading frame of conF is divided into three exons by two introns and encodes a putative protein of 91 amino acids. The deduced amino acid sequence of ConF consists of two conserved domains of CON-6 superfamily organized in a tandem fashion (Fig. 1B). The open reading frame of cffA (conF family gene A) is divided into three exons by two introns and encodes a putative protein of 162 amino acids with three conserved CON-6 superfamily domains (Fig. 1B). A comparative ClustalW alignment of the deduced amino acid sequences of various CON-6 homologs showed that the N-terminal CON-6 superfamily domain of CffA is the least conserved domain (Fig. 1C). Partial alignment of only

the N-terminus (40 aa) where the Con-6 domain is present further support that the CffA N-terminus differs from the other Con-6 like proteins.

An intron divides the open reading frame of conJ into two exons which encode a putative protein of 82 amino acids. The deduced amino acid sequence of ConJ possesses no conserved motifs in the NCBI database but is highly conserved with CON-10 homologs of other filamentous fungi (Fig. 1D). N. crassa CON proteins are hydrophilin-like proteins that have high glycine content and were proposed to be important during water deficit (Garay-Arroyo et al., 2000). Glycine constitutes more than 8% of amino acid composition in hydrophilins, resulting in higher than 1.0 hydrophilicity indexes. A comparison of glycine contents of the Con-like proteins revealed 8.7% and 15.8% glycine contents for ConF and ConJ, respectively. In contrast, CON-6 like CffA exhibits a reduced glycine fraction of only 3.7%. Glycine residues of ConF, CffA or ConJ are conserved within the fungi (Fig. 1C and D). These results suggest that the conserved Con proteins have redundant functions as hydrophilin-like proteins relevant for environmental conditions.

3.2. conF and conJ expressions are induced by short light exposure

We analyzed whether conF and conJ are induced in vegetative mycelia by light similar to con-6 and con-10 of N. crassa. Mycelia of developmentally competent culture (19 h liquid surface culture in the dark after inoculation) were exposed to light of different durations (5–120 min) to investigate conF and conJ regulation by short light exposure. RNAs were isolated and subjected to Northern hybridization analyses. conF was induced by 5 min light exposure and gradually down-regulated by longer exposure. conF was only weakly expressed after 60 min exposure. In contrast, conJ showed a complex expression pattern. conJ was expressed at significant levels after 19 h of cultivation without light exposure. conJ was induced quickly by 5 min light exposure and gradually decreased until 20 min (Fig. 2A). conJ mRNA accumulated again at 40 min and 120 min, resulting in a rhythmic expression profile.

In order to investigate the regulation of expression of conF and conJ during asexual development, RNA was extracted from synchronously differentiating asexual cultures at different time points. conF was strongly expressed in the conidiation stage (24 h culture after induction of asexual development) and faintly expressed in 24 h dark control culture. In contrast, conJ was weakly expressed in the 2 h, 4 h, 6 h and 8 h cultures before conidiation occurred, then shortly disappeared in 12 h culture. In conidiation stage (24 h culture), conJ was strongly expressed. conJ expression in 24 h dark control cultures was weak but stronger than conF levels (Fig. 2B). The short-time light responses of other light inducible genes, containing several candidates of hydrophilin-like proteins showed strong similarities to the expression profile of conF (Fig. 2C). Most of the genes were highly expressed after 5, 10 min of light exposure. cffA was expressed constitutively (data not shown).

These expression studies show that the instant expression of conF or conJ genes are controlled by short-time illumination and long-term control is coordinated with induction of asexual development.

3.3. ConF and ConJ GFP fusion localize to the cytoplasm and partially nucleus

Localization of CON-6 and CON-10 protein in N. crassa was observed by Western hybridization and immunofluorescence microscopy (Springer et al., 1992; White and Yanofsky, 1993)

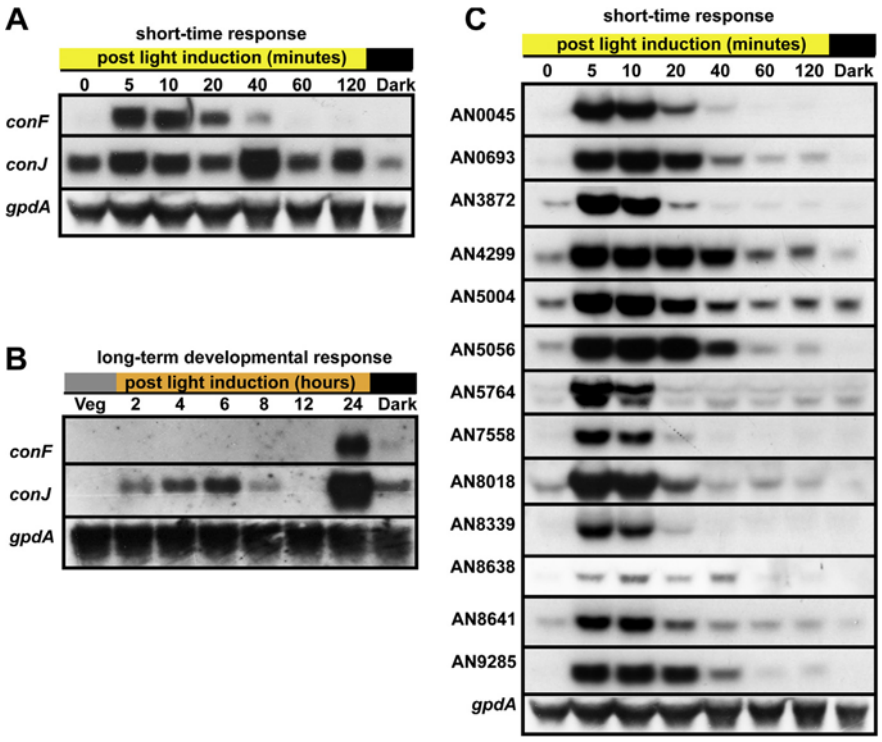

Fig. 2. Regulation of con gene expression in response to light and development. (A) Expression of conF and conJ in A. nidulans in response to short-time light exposure. A wild type strain was pre-grown on liquid surface culture for 19 h in dark were exposed to light for 5, 10, 20, 40, 60 and 120 min, then mycelia were harvested and analyzed by Northern hybridization analysis using digoxigenin (DIG) labeled probes specific to the coding regions of the conF, conJ and glycolytic gene, gpdA, served as an internal control. (B) Developmental regulation of conF and conJ in A. nidulans after onset of asexual development. Vegetative cultures were grown in dark in shaking liquid medium for approximately 20 h at 37 °C. Mycelia were harvested and transferred onto solid medium in the light to induce the asexual development. At indicated time points mycelia were harvested and subjected to Northern hybridization analysis using gene specific probes. (C) Expression of light inducible genes in A. nidulans in response to short-time light exposure. DIG labeled probes specific to the coding region of indicated genes were used for Northern hybridizations (due to low expression AN9310 was omitted among fourteen genes).

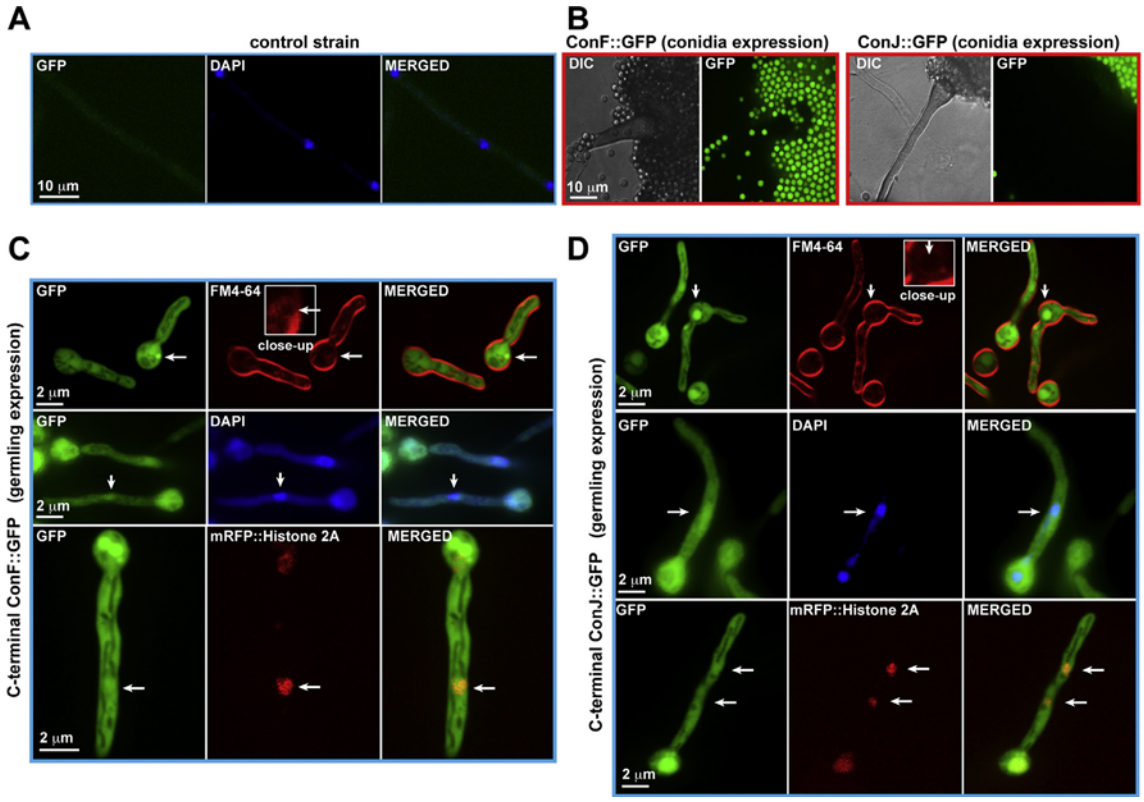

Fig. 3. Subcellular localization of Con::GFP fusion proteins in conidia and germlings. (A) A picture of recipient control strain through GFP and DAPI filters without any fluorescence marker. (B) Localizations of C-terminal ConF::GFP and ConJ::GFP in conidiophores. Both proteins are observed in mature conidia and not in conidiophore stalks. (C and D) Subcellular localization of ConF::GFP and ConJ::GFP in germlings. FM 4-64 dye stains the plasma membrane and vacuoles red. DAPI (4′,6-diamidino-2-phenylindole) stains nuclei blue and a mRFP::Histone2A fusion visualizes the nuclei positions. The granular structures of both proteins were inside the vesicle membrane. The arrow heads either show granular accumulation of GFP fusions or the positions of nuclei.

and both proteins were detected in free conidia. Functional fusions consisting of genomic DNA fragments of both conF and conJ genes containing the entire promoter regions and corresponding ORFs combined with cDNA of the green fluorescent protein (GFP) were constructed to determine cellular and subcellular localization of conF and conJ gene products (Fig. S1). Fluorescence microscopy revealed that both ConF and ConJ proteins localized in the cytoplasm of mature or free conidia but not in the conidiophore stalk (Fig. 3B). In dormant conidia, both proteins uniformly localized in the entire cytoplasm. In contrast, fluorescence of GFP was not uniform in the cytoplasm of swelling or germinating conidia resulting in occasional small bright dots of granular structure. Membranes stained by FM 4-64 revealed that granular structures of both proteins were in contact with membrane vesicles, which are presumably vacuoles (arrow head) (Fig. 3C and D). Nuclei were stained by DAPI (blue) to determine colocalization of distinct Con proteins with the nucleus. The green fluorescence signal of ConF::GFP was observed to colocalize or closely localize with the blue fluorescence signal of DAPI, suggesting association of ConF with nuclei (arrows) (Fig. 3C and D middle panel). A monomeric red fluorescent protein fused to histone 2A also colocalized to ConF protein, supporting the observations with DAPI staining (Fig. 3C lower panel).

Green fluorescence of ConJ::GFP, although in a lesser extent, was also associated with nuclei (Fig. 3D, lower panel). In late vegetative stage, Con-GFP fusions were weakly visualized due to the presumably decreased expression of the genes. In order to determine why GFP fluorescence diminishes, we monitored the ConF and ConJ protein levels in germinating conidia (Fig. 4). Both proteins were enriched in dormant conidia, whereas after 12 h post-germination Con protein levels declined. This reduction was more evident for ConF protein levels. GFP protein under the constitutive gpdA promoter was equally present during germination stages. We investigated how light exposure influences the Con protein levels (Fig. 4B). Exposure of the dark grown surface cultures to white light resulted in an increase in the expression of ConF while ConJ protein levels slightly increased after 40–80 min exposure. These results were also similar to transcript levels of the con genes during short-term light exposure. GFP expression control was unresponsive to light treatment (Fig. 4B, lower panel). Subcellular localizations of Con proteins did not alter drastically during germination and light exposure.

Our localization studies show that Con proteins are mainly localized in dormant conidia but are also present in the cytoplasm of germlings where they can colocalize with vacuole-like structures and partially with nuclei. These data suggest that Con proteins are gradually degraded during the germination process and that light exposure induces an increase in subcellular levels of Con proteins.

3.4. The double and triple con gene disruptants showed resistance to desiccation stress

In N. crassa, inactivation experiment of con-6 or con-10 did not show any clear phenotype (Springer et al., 1992; White and Yanofsky, 1993). In the genome sequence of N. crassa, there are three homologous genes of con-6 and three homologous genes of con-10. We sequentially deleted all of the three con genes of A. nidulans by the Latour system (Hirashima et al., 2006) to address the cellular function of the A. nidulans con genes (Fig. 5A). We made DconF, DcffA, DconJ mutants, but single mutants did not show any obvious phenotype (data not shown). Therefore, we created DconF/DcffA, DconF/DconJ double and Dcon triple disruptant strains and con-

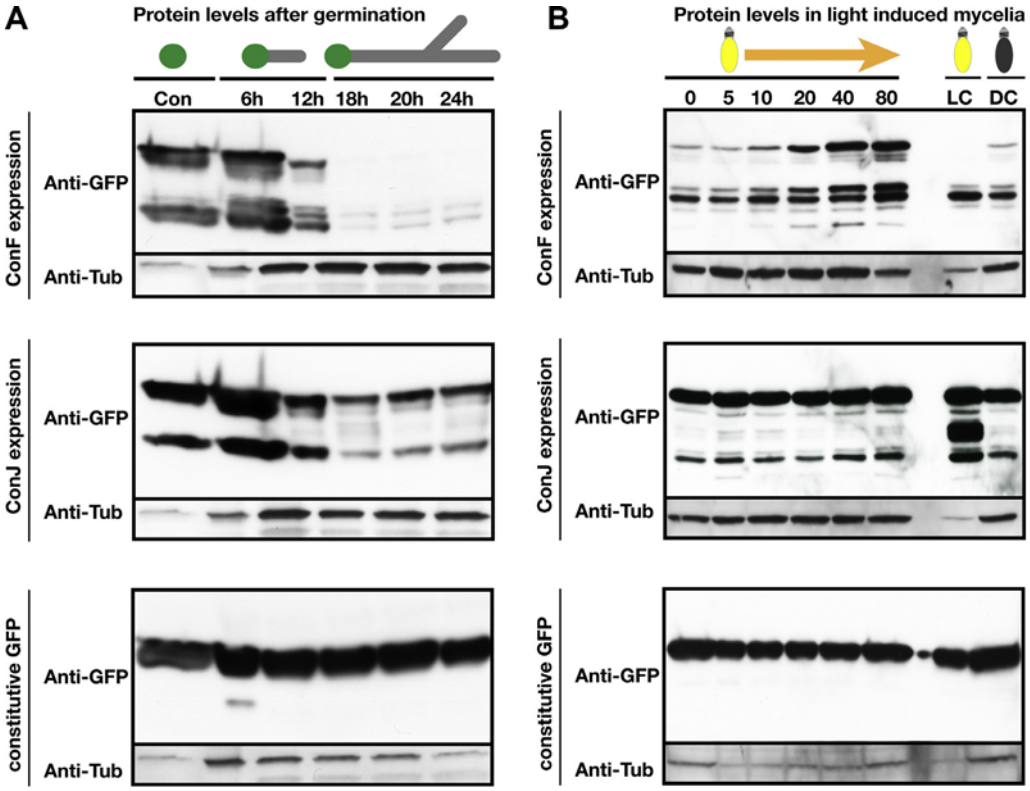

Fig. 4. Expression of ConF and ConJ::GFP fusion proteins in germinating and light induced fungal mycelia. (A) Expressional analysis of ConF and ConJ::GFP fusion proteins in post-germination phase. Con: dormant conidia, 6 h, 12 h, 18 h, 20 h, 24 h after germination at 37 °C. GFP protein expressed under constitutive promoter gpdA was used as expressional control. Tubulin protein levels were used as loading control. 40 μg protein extract was loaded on each lane. (B) Expression of ConF and ConJ::GFP fusion proteins after light induction. Strains were grown in the darkness for 24 h at 37 °C and exposed to light up to indicated time points (minutes). LC: light control cultures grown under constant light, DC: dark control cultures grown in constant darkness.

firmed the deletion of each gene by PCR (Fig. 5B). All strains grew normally as vegetative mycelia and developed normal asexual and sexual structures on solid media.

conF and *conJ* genes of *A. nidulans* are highly expressed and proteins accumulated in mature conidia. Therefore, we tested the effect of several stress conditions for conidia viability. We applied high salt or sugar concentrations adjusted to 0.9–0.94 water activity, 50 °C heat shock, oxidative stress by H_2O_2 or menadione, and UV irradiation, respectively. Wild type and single or double mutants were indistinguishable during osmotic stress, increased temperature, oxidative stress and UV irradiation (data not shown). Conidia of the Δ*con* triple disruptant strain kept 5 days at 37 °C after desiccation showed increased viability in comparison to the wild type (Fig. 5C). The conidial germination of Δ*conF*/*conJ* double and Δ*con* Triple disruptant strains were delayed for the first 4–6 h in comparison to wild type, after 8 h both strains reached the same level of germination ratio (Fig. 5D and E). These phenotypes were complemented by in-locus introduction of either *conF* or *conJ* into double deletion strain.

Germination of desiccated conidia of the Δ*conF*/*conJ* double and Δ*con* Triple disruptant strains (kept for 5 days at 37 °C after desiccation) were higher than wild type (Fig. 5F). *A. nidulans* is known to accumulate polyols upon desiccation stress including glycerol and erythritol (Beever and Laracy, 1986). We analyzed the polyols and trehalose amounts of conidia to elucidate the reason why the *con* disruptant showed higher resistance to desiccation (Fig. 5G). The Δ*conF*/*conJ* double and Δ*con* triple disruptant strain accumulated significantly higher amount of glycerol or erythritol than wild type. There was no difference in mannitol, trehalose and arabitol levels among the double, triple mutant and wild type. These data suggest that *con* genes interfere with the accumulation of specific polyols in conidia which are relevant for survival under desiccation conditions.

4. Discussion

con Genes are widely conserved in the fungal kingdom. We have shown that *con* genes in the filamentous fungus *A. nidulans* have redundant functions. This is supported by the findings that single deletions of the *con* genes do not cause obvious phenotypes, whereas simultaneous inactivations of two or three *con* genes result in delays in spore germination. This is connected with increased polyol accumulations of these mutants which correlates with increased survival of corresponding mutant strains in a dry environment. The exact molecular mechanism of the action of Con proteins is yet unclear.

The expressions of *con* genes are strictly regulated spatiotemporally according to the developmental stage or light exposure in *N. crassa* (Roberts et al., 1988; Sachs and Yanofsky, 1991; Springer and Yanofsky, 1992; White and Yanofsky, 1993) and *A. nidulans* (Fig. 2). In *N. crassa*, the mRNA accumulation of both *con-6* and *con-10* upon short light exposure increases time-dependent manner at least up to 120 min (Olmedo et al., 2010), whereas *A. nidulans conF* showed rapid adaptation and declined mRNA level according to the time of light exposure. *conJ* showed a complex expression pattern of repeated induction and repression during the first 2 h (Fig. 2A). The expression of *A. nidulans cffA* was constitutive and not controlled by light. Northern hybridization analyses of the other fourteen light inducible genes revealed a similar pattern as for *conF*

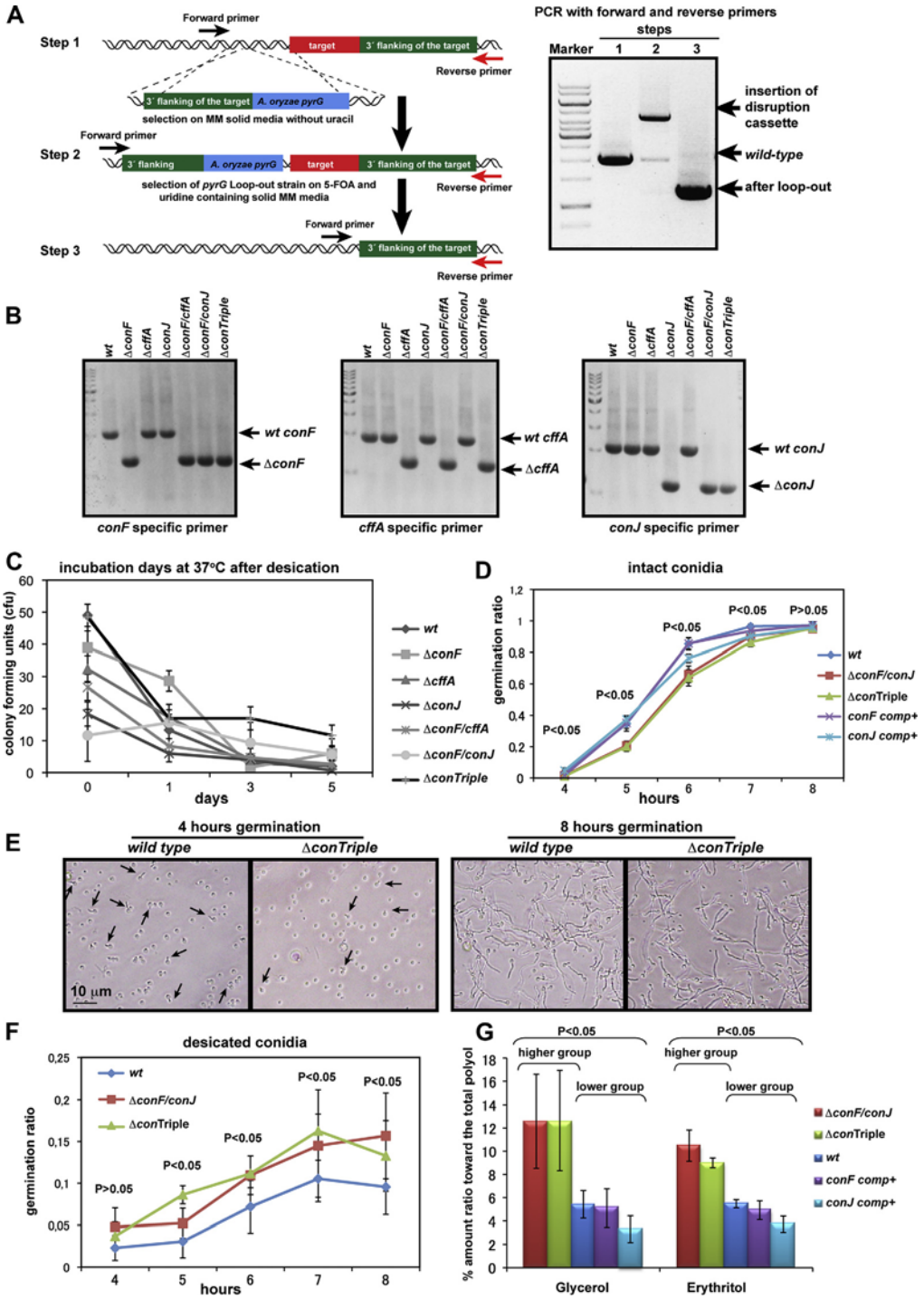

Fig. 5. Increased desiccation resistance and polyol accumulation in conF double and triple mutants. (A) Gene deletion strategy by Latour system. Band shift of the PCR amplicon shows insertion of disruption cassette and loop-out event. (B) PCR confirmation of gene disruptions for single, double and triple conF gene deletions. (C) Desiccated conidia of ΔconFTriple disruptant showed higher viability and germination ratio after 5 days incubation. 10^4 conidia of each strain were rapidly desiccated by centrifugal vaporizer and incubated at 37 °C for 0, 1, 3 and 5 days. Then conidia were rehydrated and spread on solid media at 200 conidia/plate. cfu; colony forming unit. (D) Germination ratio of intact conidia. Conidia were inoculated in liquid medium and incubated at 37 °C for 8 h. Germination ratio was calculated by dividing the number of germinating conidia by total number of conidia. (E) Microscopic image of germination from intact conidia. Arrow heads show early stage of germlings. (F) Germination ratio of conidia incubated at 37 °C for 5 days after rapid desiccation. 10^6 Conidia of each strain were rapidly desiccated by centrifugal vaporizer and incubated at 37 °C for 5 days then inoculated in liquid medium and incubated at 37 °C for 8 h. The P values represent the significance between wild type and two mutant strains. (G) Polyol content of conidia. polyols were extracted from each strain and analyzed by HPLC. The P values (<0.05) were calculated by t-test between higher group (double and triple mutant) and lower group (wild type and complementation strains).

except for AN9310 where expression was too weak to evaluate (Fig. 2C). Except for the complex conJ regulation, the typical light expression pattern of *A. nidulans* light inducible genes show more rapid induction and adaptation than *N. crassa* con-6 and con-10. The exact mechanism of this quick induction and photoadaptation is not clear. *N. crassa* shows photoadaptation by longer illumination, usually 1–2 h, which depends on the VVD protein (Schwerdtfeger and Linden, 2001). There is no obvious homolog

Table 4
Hydrophilicity profile of light-induced genes.

Gene ID	Protein size (a.a.)	Preponderance amino acid residues in Hydrophilin (%)						Low proportion aa in Hydrophilin		Hydropathy profile
		Gly	Ala	Glu	Lys	Arg	Thr	Cys	Trp	Values Hydrophilicity + values Hydrophobicity
AN0045	378	9.8	11.6	5.8	7.4	3.2	9.5	0.0	0.3	
AN 0693	136	7.4	11.8	5.1	5.1	5.1	7.4	0.0	0.7	
AN3872	245	7.8	9.11	7.3	4.9	2.9	9.4	0.8	3.7	
AN4299	151	8.6	17.2	3.3	3.3	3.3	9.3	0.0	2.0	
AN5004	147	10.2	19.7	6.8	10.2	6.1	4.8	0.0	0.7	
AN5015 (ConJ)	82	15.9	11.0	11.0	4.9	7.3	1.2	0.0	0.0	
AN5056	197	9.6	16.9	8.5	14.1	1.4	11.3	0.0	0.0	
AN5764	775	5.3	14.3	10.1	4.8	10.5	5.2	0.0	0.4	
AN5844	162	3.7	11.7	13.0	4.9	12.3	4.3	0.0	0.0	
AN7558	197	9.6	7.6	8.6	5.1	8.1	8.1	0.0	0.0	
AN8018	445	8.1	8.3	3.6	6.3	3.1	6.5	1.3	1.6	
AN8339	405	8.6	10.4	7.9	11.9	3.5	4.4	0.2	1.7	
AN8638	228	2.2	4.4	8.8	7.9	5.7	6.6	0.0	1.3	
AN8640 (ConF)	91	8.8	11.0	8.8	8.8	5.5	2.2	0.0	0.0	
AN8641	131	9.2	9.2	5.3	6.1	6.9	10.7	0.0	0.8	
AN9285	71	2.8	19.7	7.0	15.5	1.4	7.0	0.0	0.0	
AN9310	158	8.2	10.1	7.0	5.7	13.3	5.7	0.0	0.0	

of VVD in A. nidulans (Rodriguez-Romero et al., 2012). It is unknown for A. nidulans whether another protein plays a VVD-like role or whether there exists a different mechanism for photoadaptation.

Phenotypes of double and triple disruptant strains of con genes interfered with spore viability, germination and polyol accumulation (Fig. 5). Glycerol and erythritol are major osmoregulatory compounds in A. nidulans, whereas arabitol and mannitol do not represent important osmoregulators (Beever and Laracy, 1986). Trehalose or mannitol are normally accumulated during heat (Noventa-Jord et al., 1999) or oxidative stress (Fillinger et al., 2001). The double and triple disruptants accumulated more glycerol and erythritol (Fig. 5G). In contrast, cffA had no significant additional impact on polyol accumulation or germination ratio. Deletion of several con genes might induce an osmotic stress response, which results in the accumulation of polyols. This suggests a possible redundant control function of con genes in the adaptation of cellular polyol levels. Redundant functions are also found for other sporulation related genes where even the deletion of the entire A. nidulans SpoC1 cluster resulted in no phenotype (Aramayo et al., 1989; Stephens et al., 1999). Further deletions of con-like genes might achieve even stronger phenotypes.

Ruger-Herreros and co-workers showed that 425 light inducible genes are unevenly distributed in the different chromosomes. It is unclear whether even proteins without obvious similarity might exhibit similar functions. CON-6 is structurally similar to the late embryogenesis abundant proteins (LEA) of maize. LEA proteins did not evolve from a common ancestral protein (Garay-Arroyo et al., 2000) and cannot be recognized by primary sequence alignment. Garay-Arroyo et al. established a search criterion to distinguish LEA from other proteins based on physicochemical features of the polypeptide. They coined the name "hydrophilins" to proteins defined by their criterion. They selected CON-6 and CON-10 from 2264 fungal sequences as hydrophilin candidates. ConF and ConJ and most of the light inducible gene products as well as the constitutively expressed CffA might be hydrophilins (Table 4).

Structural features of the LEA proteins as a subset of hydrophilins include high hydrophilicity, a lack or low proportion of Cys and Trp residues, and a preponderance of Gly, Ala, Glu, Lys/Arg, and Thr (Dure, 1993). We analyzed the structural features of seventeen fungals proteins including ConF, CffA and ConJ (Table 4). Due to amino acid composition analyses for hydrophilicity Cys and Trp residues content seems to be more important than Gly residues. From this aspect, CffA may be classified as hydrophilin even though it has a low Gly content. Con proteins and other hydrophilin-like proteins might have functions during water deficiency.

In N. crassa, CON-6 and CON-10 accumulate in mature conidia and are degraded during germination (Springer et al., 1992; White and Yanofsky, 1993). A. nidulans ConF and ConJ proteins also localized in mature conidia (Fig. 3A). They are not present in conidiophores or immature conidia and are also degraded during germination (Fig. 4A). In mature conidia, ConF and ConJ are uniformly dispersed in the cytosol, whereas in germinating conidia, they aggregate as granular structures in the cytolasm. Some of these granular structures of ConF or ConJ in germinating conidia are associated with membrane vesicles (Fig. 3C and D).

There is a colocalization of ConF and ConJ with nuclei, which indicates that there might be an additional yet unknown nuclear function. This is reminiscent to some spore proteins of Bacilli, which have been described as small acid-soluble proteins (SASPs) that bind unspecifically to DNA. Such proteins protect spore DNA from various stresses (Setlow and Setlow, 1994, 1995). It remains to be analyzed whether Con proteins have similar functions as bacterial spore proteins to protect genomic DNA within the nuclei.

5. Conclusion

Con proteins are similar to hydrophilins. Expression of the Con encoding genes conF and conJ of A. nidulans is strictly regulated by short time exposure of light during development. Con proteins exhibit redundant functions in spore germination and in controlling the level of stress protection in germinating conidia.

Acknowledgments

This study was supported by the studying-abroad program of the National Agriculture and Food Research Organization, Japan to Satoshi Suzuki and by the Deutsche Forschungsgemeinschaft (DFG) and the SFB860 to GHB.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2013.04.008.

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