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

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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 phytocrome 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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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 μW 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 Δ*conF* intermediate strain (*pyrG*+). Conidia of the Δ*conF* strain (*pyrG*+) were inoculated on MM solid media with 5-FOA and uridine. 5-FOA resistant Δ*conF* strain (*pyrG*−) was selected (Table 1). To make *cffA* deletion construct, 5UTR region, 3UTR 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 Δ*cffA* intermediate strain (*pyrG*+). 5-FOA resistant Δ*cffA* strain (*pyrG*−) was obtained as explained above. *conJ* deletion construct was made by amplifying 5UTR (SAT14/109) and 3UTR (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 Δ*conJ* strain (*pyrG*+). Δ*conJ* strain (*pyrG*−) was obtained on 5-FOA media. *cffA* deletion cassette was transformed into Δ*conF* strain (*pyrG*−) to give intermediate Δ*conF*/Δ*cffA* strain (*pyrG*+). Δ*conF*/Δ*cffA* strain (*pyrG*−) was selected on 5-FOA media. *conJ* deletion construct was transformed into Δ*conF* strain (*pyrG*−) to yield intermediate Δ*conF*/Δ*conJ* strain (*pyrG*+) and Δ*conF*/Δ*conJ* double deletion (*pyrG*−). *cffA* deletion cassette was introduced into Δ*conF*/Δ*conJ* double deletion (*pyrG*−), which led to Δ*conF*/Δ*conJ*/Δ*cffA* 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 *Kpn*I site. This *Kpn*I site can ligate with the *Kpn*I 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* 5UTR (OSBS11/13), *conF* ORF (OSBS14/15), *conF* 3UTR (OSBS16/17) were amplified from the wild type genomic DNA. 5UTR, *gfp*, *conF* ORF, *pyroA*, 3UTR fragments were cloned in *Sma*I site of pUC19 by using in-Fusion cloning kit (Clontech) pOB325. Similarly, *conJ* 5UTR (OSBS19/21), *gfp*, *conJ* ORF (OSBS22/23), *pyroA*, *conJ* 3UTR (OSBS24/25) were joined and cloned (pOB326).

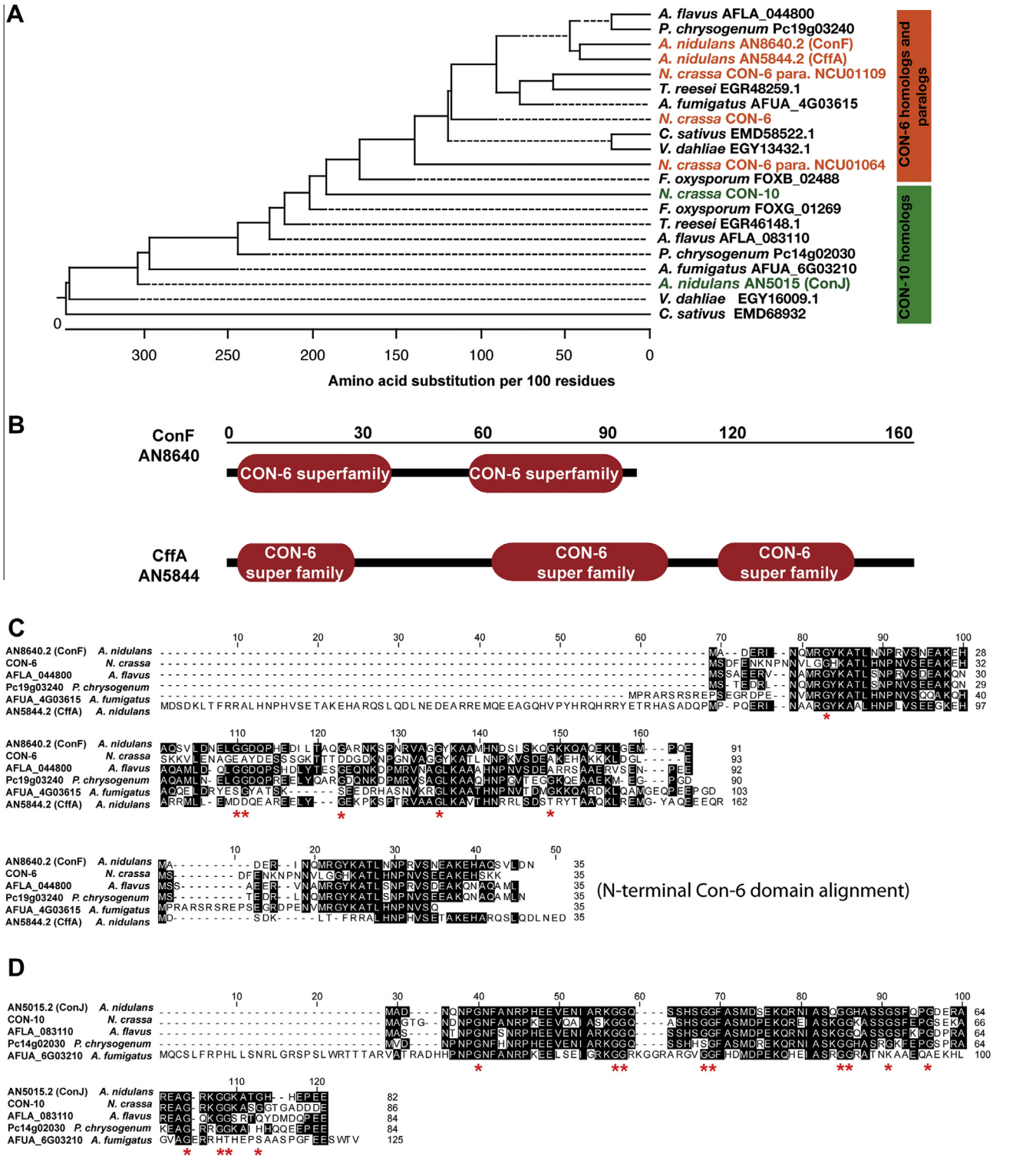

Fig. 1. Phylogenetic tree and domains found in Con proteins. (A) The phylogenetic tree of Con homologous proteins. The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987). Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). *Neurospora crassa* CON-6 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	*nkuAΔ::argB, pyrG89, pyroA4, veA+*	Bayram et al. (2012)
AGB552	*nkuAΔ::argB, pabaA1, veA+*	Bayram et al. (2012)
ΔconF	*conFΔ, nkuAΔ::argB, pyrG89, pyroA4, veA+*	This study
ΔcffA	*cffAΔ, nkuAΔ::argB, pyrG89, pyroA4, veA+*	This study
ΔconJ	*conJΔ, nkuAΔ::argB, pyrG89, pyroA4, veA+*	This study
ΔconF/ΔcffA	*conFΔ, cffAΔ, nkuAΔ::argB, pyrG89, pyroA4, veA+*	This study
ΔconF/ΔconJ	*conFΔ, conJΔ, nkuAΔ::argB, pyrG89, pyroA4, veA+*	This study
Δcon triple	*conFΔ, cffAΔ, conJΔ, nkuAΔ::argB, pyrG89, pyroA4, veA+*	This study
conF GFP	*conF::egfp-pabaA; nkuAΔ::argB, pabaA1, veA+*	This study
conJ GFP	*conJ::egfp-pabaA; nkuAΔ::argB, pabaA1, veA+*	This study
N GFP conF	*N-gfp::conF, AfpyroA, nkuAΔ::argB, pyroA4, pyrG89, veA+*	This study
N GFP conJ	*N-gfp::conJ, AfpyroA, nkuAΔ::argB, pyroA4, pyrG89, veA+*	This study
conF complementation	5 UTR *conF-pyroA* 3 UTR ΔconF/ΔconJ	This study
conJ complementation	5 UTR *conJ-pyroA* 3 UTR ΔconF/ΔconJ	This study
E. coli		
MACH-1	*F-φ80(lacZ)ΔM15ΔlacX74 hsdR(rK–mK +)ΔrecA1398 endA1 tonA*	Invitrogen
DH5α	F^-, *Φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17*(r_K^-, m_K^+), *phoA, supE44,* λ^-, *thi-1, gyrA96, relA1*	Hanahan (1983)

Table 2
Plasmids employed in this study.

Plasmid	Description	Reference
pJET 1.2	Cloning plasmid	Fermentas
pUC19	Cloning plasmid	Fermentas
pOB325	*N-gfp::conF-pyroA* in *Sma*I site of pUC19	This study
pOB326	*N-gfp::conJ-pyroA* in *Sma*I site of pUC19	This study
pOB327	5 UTR *conF-pyroA* 3 UTR in *Sma*I site of pUC19	This study
pOB328	5 UTR *conJ-pyroA* 3 UTR in *Sma*I 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 5UTR and *conF* ORF (OSBS11/15), *conF* 3UTR (OSBS16/18), which were fused to *pyroA* by PCR (OSBS11/18) and cloned into *Sma*I site of pUC19 (pOB327). pOB327 served as a template for amplification of complementation cassette with primers OSBS12/17. Likewise, 5UTR and *conJ* ORF (OSBS19/23), *conj* 3UTR (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 μg 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 μl 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 μl 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 23,000*g* and flowthrough containing polyols and sugars were collected. Before use, Microcon cartridges were washed by total 3 l H_2O for two over night 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, mannnitol 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 *Kpn*I
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 *Kpn*I
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 *pyrG5*′
SAT100	TGC TCT CGT AGA GAC CAA AGA TGG ATT CTG ACA AGC TGA CCT TC	*cffA* deletion ORF 5′ primer with *pyrG3*′
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 *pyrG5*′
SAT107	TGCTCTCGTAGAGACCAAAGTCTTGCGCCTGCTTTTTCCCCT	*conF* deletion ORF 5′ primer with *pyrG3*′
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 *pyrG5*′
SAT112	TGC TCT CGT AGA GAC CAA AGG AAA CTT TTG GCC CTT TTT AGC ATG CC	*conJ* deletion ORF 5′ primer with *pyrG3*′
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	*conJ* 5UTR pUC19 fusioner
OSBS20	GCA TCT GGT GAC GAG CAT AGC	*conJ* 5UTR nest
OSBS21	CGC CCT TGC TCA CCA TGA TGT ATT TAA AG AAT TGG TTG TGG	*conJ* 5UTR 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	*conJ* 3UTR nest
OSBS25	CCA CAG GAA TCA ATA CAA CCG	*conJ* 5UTR pUC19 fusioner
OSBS26	TCT AGA GGA TCC CCC CAC AGG AAT CAA TAC AAC CG	*conJ* 5UTR nest

(Shodex) using an isocratic elution with CH_3CN/H_2O (75/25) at 1.0 ml min^{-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)

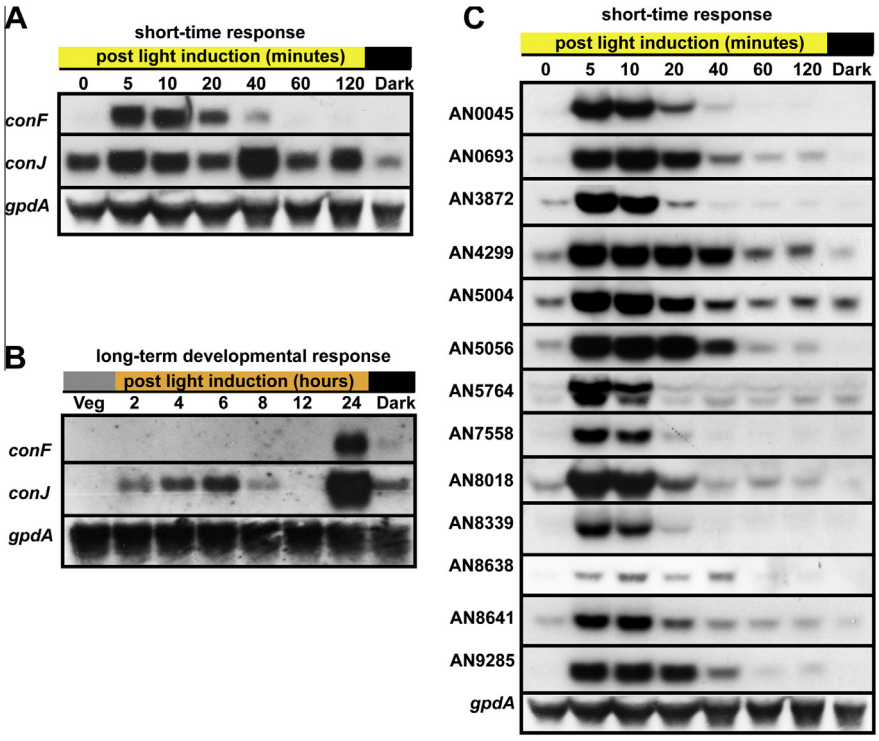

Fig. 2. Regulation of *con* gene expression in response to light and development. (A) Expression of *conF* and *conJ* in *A. nidulans* in response to short-time light exposure. A wild type strain was pre-grown on liquid surface culture for 19 h in dark were exposed to light for 5, 10, 20, 40, 60 and 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).

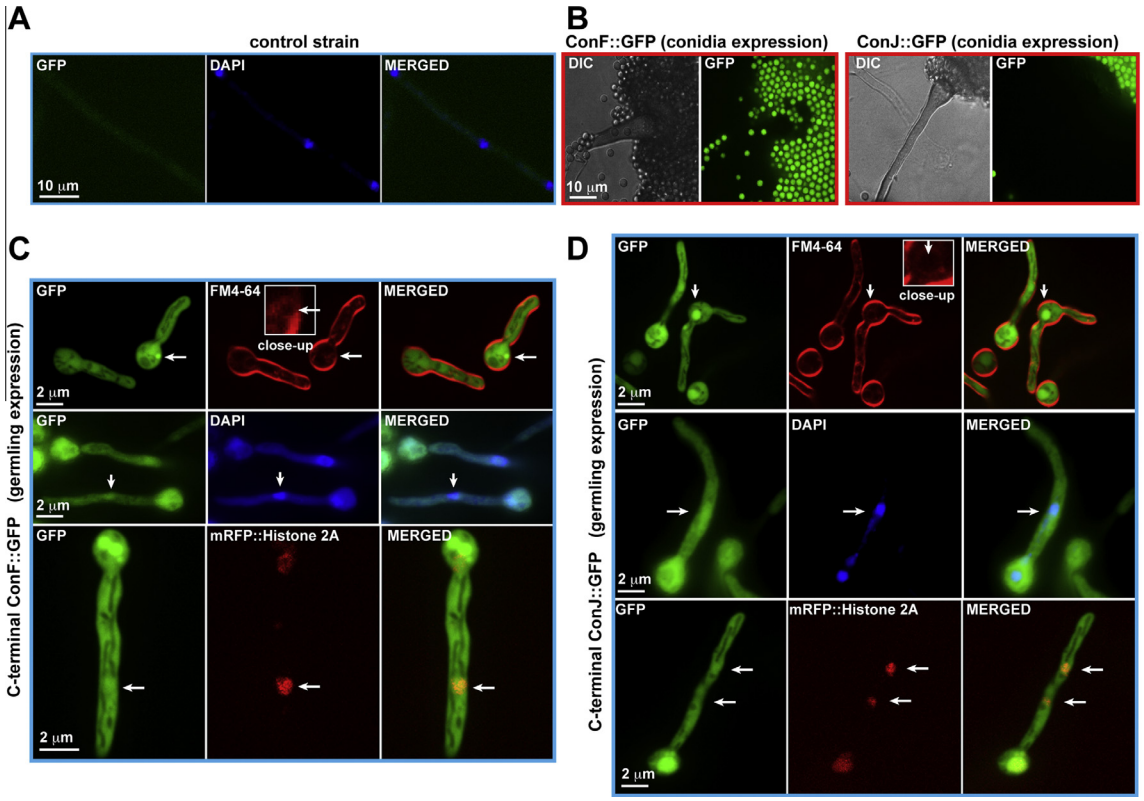

Fig. 3. Subcellular localization of Con::GFP fusion proteins in conidia and germlings. (A) A picture of recipient control strain through GFP and DAPI filters without any fluorescence marker. (B) Localizations of C-terminal ConF::GFP and ConJ::GFP in conidiophores. Both proteins are observed in mature conidia and not in conidiophore stalks. (C and D) Subcellular localization of ConF::GFP and ConJ::GFP in germlings. FM4-64 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 FM4-64 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 Δ*conF*, Δ*cffA*, Δ*conJ* mutants, but single mutants did not show any obvious phenotype (data not shown). Therefore, we created Δ*conF*/Δ*cffA*, Δ*conF*/Δ*conJ* double and Δ*con* triple disruptant strains and con-

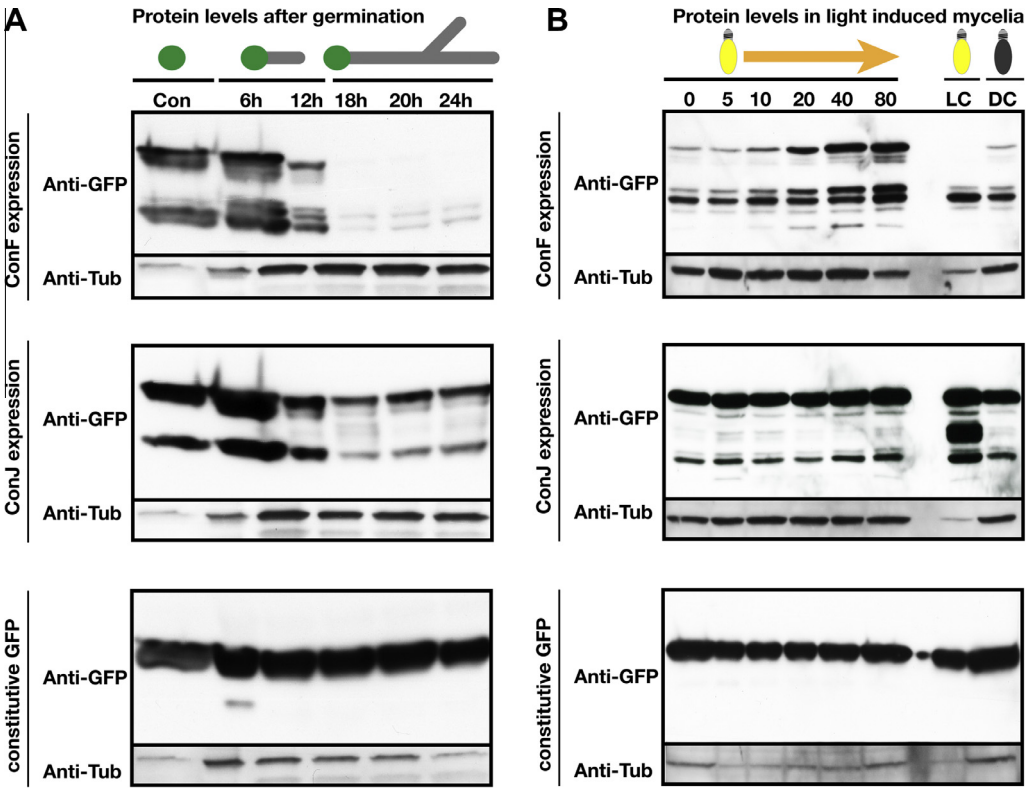

Fig. 4. Expression of ConF and ConJ::GFP fusion proteins in germinating and light induced fungal mycelia. (A) Expressional analysis of ConF and ConJ::GFP fusion proteins in post-germination phase. Con: dormant conidia, 6 h, 12 h, 18 h, 20 h, 24 h after germination at 37 °C. GFP protein expressed under 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*

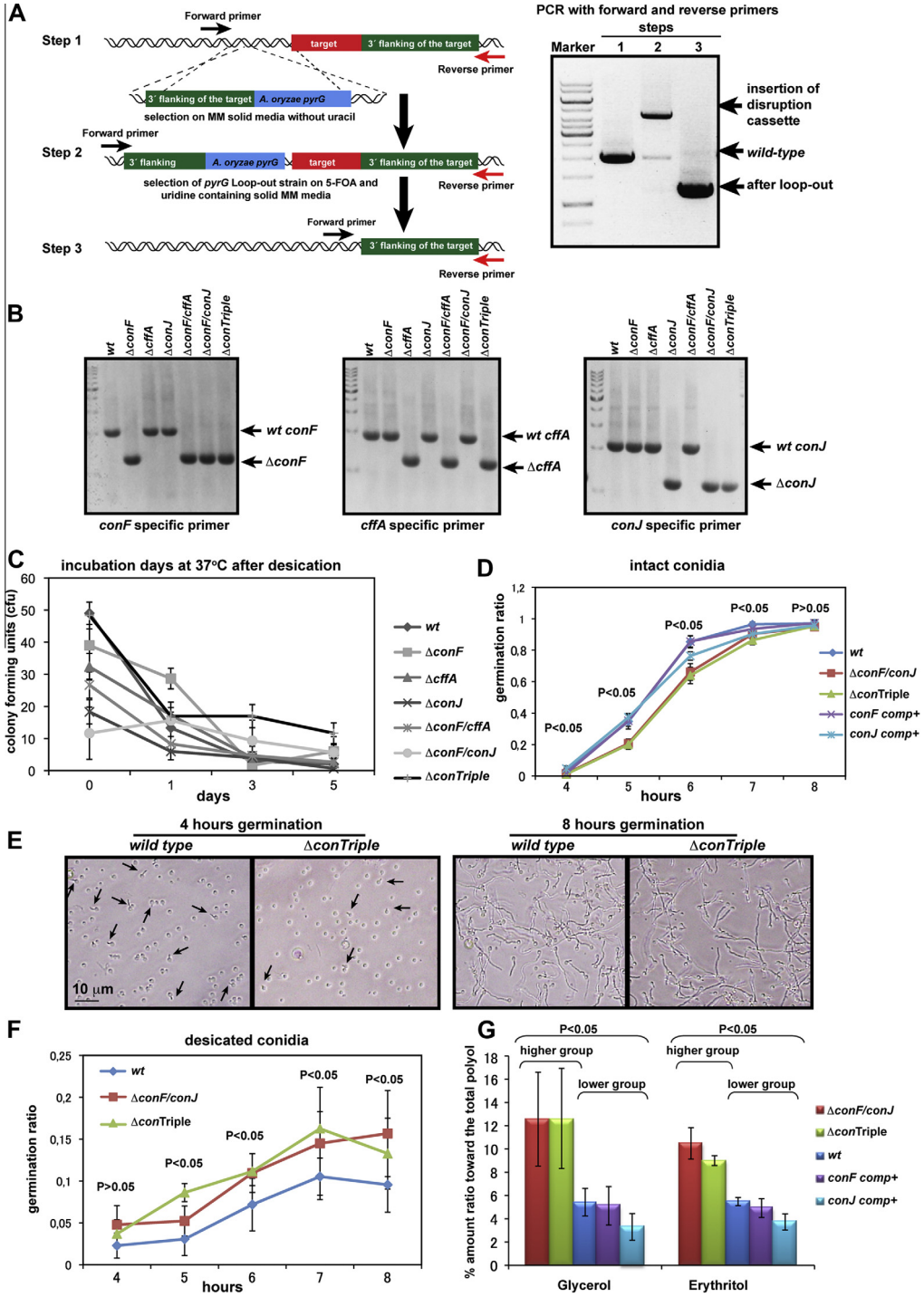

Fig. 5. Increased desiccation resistance and polyol accumulation in *con* double and triple mutants. (A) Gene deletion strategy by Latour system. Band shift of the PCR amplicon shows insertion of disruption cassette and loop-out event. (B) PCR confirmation of gene disruptions for single, double and triple *con* gene deletions. (C) Desiccated conidia of ΔconTriple 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 unkwnown 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.

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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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