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Cpc1 mediates cross-pathway control independently of Mbf1 in *Fusarium fujikuroi*

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ABSTRACT

The deletion of *glnA*, encoding the glutamine synthetase (GS), had led to the down-regulation of genes involved in secondary metabolism and up-regulation of *cpc1*, the cross-pathway control transcription factor. In the present study, a Δ*cpc1* mutant was created and used for transcriptional profiling by macroarray analysis. Most of the Cpc1 target genes were amino acid biosynthesis genes besides a homologue of the multi-protein bridging factor MBF1 that binds to the yeast Cpc1 homologue GCN4. We show that Δ*mbf1* mutants exhibit no Cpc1-related phenotype and that both proteins do not interact with each other in *Fusarium fujikuroi*. Moreover, results presented here suggest that Cpc1 is not responsible for the GS-dependent down-regulation of secondary metabolism and that its role is focused on the activation of amino acid biosynthesis in response to the amino acid status of the cell. Surprisingly, cross-pathway control is repressed by nitrogen limitation in an AreA-dependent manner.

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1. Introduction

The heterothallic ascomycete *Fusarium fujikuroi* (teleomorph *Gibberella fujikuroi* mating population C) is well known for its ability to produce gibberellins (GAs), a family of plant hormones, causing hyper-elongation of its host plant rice (Kurosawa, 1926). The production of GAs is under the control of the nitrogen metabolite repression network: the GATA type transcription factor AreA activates six of the seven biosynthesis cluster genes only under nitrogen-limited conditions (Mihlan et al., 2003; Tudzynski et al., 1999). Beside GAs, *F. fujikuroi* produces the red polyketide pigment bikaverin under nitrogen-limited conditions, but in contrast to GA biosynthesis genes, AreA is not essential for expression of bikaverin genes (Wiemann et al., 2009). The presence of nitrogen leads to an almost complete repression of GA and bikaverin biosynthesis, with glutamine being the strongest repressing nitrogen source in *F. fujikuroi* (Muñoz and Agosin, 1993). We previously showed that deletion of the *glnA* gene coding for the glutamine synthetase (GS) did not result in derepression of the GA and bikaverin genes that would be expected due to the decreased endogenous concentration of the repressor glutamine but resulted in strong repression of these secondary metabolism genes (Teichert et al., 2004). On the other hand, a set of genes was shown to be significantly up-regulated in the *glnA* mutant, among them *cpc1* and *mbf1* encoding homologues of the fungal bZIP transcription factor Cpc1 (GCN4 in yeast) and the GCN4 co-activator in *Saccharomyces cerevisiae*, MBF1. In *Neurospora crassa*, the transcription factor CPC1 is the central transcription factor of cross-pathway control, mediating the transcriptional up-regulation of genes responsible for amino acid biosynthesis under conditions of amino acid limitation (Carsiotis and Jones, 1974), which is elicited by limitation of any one of at least 16 amino acids (Barthelmess and Kolanus, 1990). Recently, a comparative analysis of the cross-pathway control regulon had been performed in *N. crassa* where it was shown that Cpc1 controls at least 443 target genes, most of them from amino acid and nucleotide metabolism, tRNA synthesis, vitamin metabolism and protein degradation (Tian et al., 2007).

In *Aspergillus nidulans*, the Cpc1 homologue CpcA has been suggested to be involved in negative regulation of penicillin biosynthesis genes (Busch et al., 2003). In this fungus, the non-proteinogenic amino acid alpha-aminoadipate defines the biosynthetic branch-point of lysine and penicillin biosynthesis. Upon amino acid limitation, *lysA* (whose gene product saccharopine dehydrogenase catalyses the ultimate step of the lysine-specific branch) is significantly up-regulated in a CpcA-dependent manner, while the expression of the penicillin-biosynthesis genes, *acvA* and *ipnA*, is drastically reduced. These data suggest that, upon amino acid starvation, the cross-pathway control overrules secondary metabolite biosynthesis and favours the metabolic flux towards amino acids instead of penicillin in *A. nidulans* (Busch et al., 2003), probably by directly repressing penicillin biosynthetic genes. In *S. cerevisiae*, the general amino acid control (the equivalent to the cross-pathway control operating in filamentous fungi) is activated not only by amino acid starvation, but also purine starvation (Mösch et al., 1991), lack of tRNA synthetases

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(Meussdoerffer and Fink, 1983), UV radiation (Engelberg et al., 1994), glucose starvation and growth on ethanol (Yang et al., 2000), rapamycin addition (Valenzuela et al., 2001), the alkylating chemical methyl methanesulfonate (Natarajan et al., 2001), high salt concentrations in the environment (Goossens et al., 2001), high concentrations of methylglyoxal (Nomura et al., 2008) and high concentrations of hydrogen peroxide (Mascarenhas et al., 2008). In response to these stimuli, the transcription of more than 500 target genes is up- or down-regulated (Natarajan et al., 2001), indicating that GCN4 can act as a transcriptional activator or repressor, respectively, by binding to GCREs (general control response elements) and regulating transcription of its target genes. For this activation, GCN4 needs the co-activator MBF1, the so-called multi-protein bridging factor, which recruits the TATA-box binding protein SPT15 to the complex of GCN4 with the promoter of target genes. When MBF1 is missing, the GCN4 target gene *HIS3* is no longer expressed under conditions of amino acid starvation (Takemaru et al., 1998).

In this work, we report on the cloning and characterization of the *F. fujikuroi* cross-pathway control transcription factor, termed Cpc1. First of all, we show that Cpc1 is not involved in unexpected down-regulation of GA and bikaverin genes in the *F. fujikuroi* Δ*glnA* mutant as it was speculated before (Teichert et al., 2004). Secondly, we identify a set of Cpc1 target genes, mainly involved in biosynthesis of amino acids, by macroarray analysis. Third, we show that a protein with similarity to the GCN4 co-activator MBF1 from *S. cerevisiae*, which was also shown to be up-regulated in the Δ*glnA* mutant, is not involved in cross-pathway control in *F. fujikuroi*, and that Cpc1 expression is repressed under conditions of nitrogen starvation and induced by starvation for, or oversupply of, a single amino acid at both transcriptional and post-translational level. Furthermore, we show for the first time that cellular localization of Cpc1 depends on the nitrogen status of the cell.

2. Materials and methods

2.1. Fungal strains and culture conditions

Strain IMI58289 (Commonwealth Mycological Institute, Kew, UK) is a gibberellin-producing wild-type strain of *F. fujikuroi*. The Δ*glnA* strain was described earlier (Teichert et al., 2004). For all cultivations, the *F. fujikuroi* strains were pre-cultivated for 48 h in Darken medium (Darken et al., 1959) with 2.0 g l^{-1} glutamine instead of $(NH_4)_2SO_4$, and 1 ml of this culture was used as inoculum for cultivations in ICI or CM media. For DNA isolation and production of protoplasts, *F. fujikuroi* strains were incubated in 100 ml complete medium (CM) (Pontecorvo et al., 1953) at 28 °C on a rotary shaker at 190 rpm for 3 days or 18 h, respectively. For RNA isolation, the fungal strains were grown for 4 days in synthetic ICI medium (Geissman et al., 1966) containing 2.0 g l^{-1} glutamine on a rotary shaker at 28 °C. The washed mycelium was transferred into ICI medium without nitrogen (0% ICI) for 4 h to induce nitrogen starvation, and then transferred for 2 h into media with or without 4 mM L-methionine-DL-sulfoximine, 40 mM 3-amino triazole, 200 ng μl^{-1} rapamycin or different nitrogen sources. Amino acid auxotrophic *F. fujikuroi* mutants were generated in a UV random mutagenesis experiment with subsequent filtration enrichment and by targeted deletion of the glutamate synthase gene *gltA* and the NADP(+)-dependent glutamate dehydrogenase gene *gdhA* (Δ*gltA*/*gdhA*-T13, Huber and Tudzynski, unpublished).

2.2. Bacterial strains and plasmids

Escherichia coli strain Top10F′ (Invitrogen, Groningen, The Netherlands) was used for plasmid propagation. Vector pUC19 was used for cloning of lambda DNA restriction fragments and products of PCR amplification were cloned into the vector pCR®2.1–TOPO® using the TOPO TA Cloning Kit (Invitrogen, Groningen, The Netherlands). The *cpc1* gene was cloned by PCR using the forward primer cpc-u-F3 (situated in the putative second uORF), and the reverse primer cpc-R3 at the end of the coding region. The resulting 1.2-kb fragment was cloned and sequenced. Additional sequence information was gained through Thermal Asymmetric Interlaced PCR (TAIL-PCR) (Terauchi and Kahl, 2000) for the 3′, and a screening of a genomic lambda library of *F. fujikuroi* for the 5′ UTR region of the *cpc1* locus. For *cpc1* gene replacement, a 0.45-kb *Sac*I-*Xba*I-fragment from the 5′-region and a 0.5-kb *Hin*dIII/*Sal*I fragment from the 3′-non-coding region were cloned into the plasmid pNR1 carrying a nourseothricin resistance cassette (Malonek et al., 2004). For creation of the *mbf1* deletion vector pΔmbf1, flanks were generated using the primers mbf-Kpn and mbf-Sal-ko for amplification of the 5′ and mbf-Hind and mbf-Bam for amplification of the 3′ flank. The 5′ flank was cloned into the *Kpn*I/*Sal*I digested pUCH2-8 vector (Alexander et al., 1998), and the 3′ flank was cloned into the *Hin*dIII/*Bam*HI digested vector, giving the vector pΔmbf1. The yeast two-hybrid vectors were prepared as follows: for the GAL4 yeast two-hybrid, 0.75-kb *cpc1* and 0.48-kb *mbf1* fragments were amplified from cDNA as template with oligonucleotides containing an *Eco*RI site for the forward (cpc–Eco, mbf–Eco) and a *Sal*I site for the reverse primer (cpc–Sal, mbf–Sal). After cloning into pCR®2.1-TOPO® and sequencing, fragments were cloned into the pAD-GAL4-2.1 (*cpc1*) or in the pBD-GAL4 Cam (*mbf1*) vector, respectively (Stratagene, La Jolla, USA), giving the vectors pAD-cpc1 and pBD-mbf1. A fragment for cloning into pSOS for the CytoTrap® two-hybrid was amplified from cDNA as template using the primers cpc_SOSfor(Nco) and cpc_SOSrev(Sal), cloned into pCR®2.1–TOPO® and sequenced, digested with *Nco*I and *Sal*I and cloned into the *Nco*I/*Sal*I digested pSOS vector (Stratagene, La Jolla, USA), resulting in the vector pSOS-cpc1. The *mbf1* cDNA insert from pBD-mbf1 was excised with *Eco*RI and *Sal*I and ligated into the *Eco*RI/*Sal*I digested pMyr vector (Stratagene, La Jolla, USA), giving the vector pMyr-mbf1.

The GFP vector pWMS was constructed the following way: *Bam*HI and *Xba*I sites of pBluescriptSK- were deleted by digestion with these enzymes, subsequent blunt ending with the Klenow fragment of DNA polymerase I (Fermentas, St. Leon-Rot, Germany) and ligation with T4 ligase (Invitrogen, Groningen, The Netherlands), giving the vector pBSΔBamXba. The *sgfp* gene under the control of the *N. crassa ccg1* promoter was amplified from vector pMF272 (Freitag et al., 2004) with the oligonucleotides ccg1-P-Sal containing an artificial *Sal*I site and GFP-Eco-Sph containing *Eco*RI and *Sph*I sites and cloned after digestion with *Eco*RI and *Sal*I into the respective sites of the pBSΔBamXba vector. The *hph* hygromycin resistance gene under the control of the *A. nidulans oliC* promoter was amplified from the pOliHP vector (Rolke et al., 2004) with the oligonucleotides OliC-for-Xho and hph-rev-Hind and cloned into pCR®2.1–TOPO® (Invitrogen, Groningen, The Netherlands). The terminator of a tubulin gene from *B. cinerea* was amplified from vector pNR1 (Malonek et al., 2004), using the oligonucleotides T-Tub-for-Hind and T-Tub-rev-Xho-Hind, and cloned into the *Hin*dIII site following the *hph* gene. The resulting vector was then digested with *Xho*I and the resistance cassette was cloned into the *Xho*I site of pBSΔBamXba containing the *ccg1* promoter and the *sgfp* gene, yielding the vector pWMS.

The vector pWMS-cpc1 was created by amplifying a 0.7-kb *cpc1* fragment with additional *Sph*I and *Eco*RI restriction sites, which was ligated into pWMS after cloning in pCR®2.1–TOPO® and sequencing. All oligonucleotides used in this study are listed in Table 1.

2.3. Screening of a genomic library

For cloning the entire genomic region of the *cpc1* and *mbf1* genes, about 40000 recombinant phages of a Lambda DASH II

Table 1
Oligonucleotides used in this study.

Oligonucleotide	Sequence
cpc-u-F3	5′-ATG GCT TCT CTC CAC CAG CCT GCC GGC AC-3′
cpc-R3	5′-TTT AAT CCA TTA TTG CAC ACC TGA TTG CGC-3′
cpc-T1	5′-GGC AAT GCG ACT ATC TCG CCC CCA AGA TC-3′
cpc-T2	5′-TGG ATA CGA TGT GTC TCC AAA CTT CGG CAG-3′
cpc-T3	5′-CTC ACC GGA GCT CAA GTC TGA TGA AGT CG-3′
cpc-T4	5′-CCG TCG CTA TGA AGC GTG CTC GAA ACA CTC-3′
cpc-T5	5′-GGA AGA GGA TCG CTC TTG CGC AAT CAG GTG-3′
RAPD-E4	5′-GTG ACA TGC C-3′
cpc-LFF	5′-GTC AAC TAC CGC GGT GCA ATT C-3′
cpc-LFR	5′-CCA GAT TGC CTC TAG AGC TG-3′
cpc-RFF	5′-GAG GAA AGC TTT TGC GCA ATC AGG TGT GC-3
cpc-RFR	5′-CAG GGC GTC GAC ACT ATG CTT ATC TCG GC-3′
cpc-LFcheck	5′-CAG AGT CCC GAA TCA CCA CAA CAG G-3′
cpc-RFcheck	5′-GAC ATG CCC ATA AGC TTA AAC CGA CG-3′
pLof-OliP	5′-GGT ACT GCC CCA CTT AGT GGC AGC TCG CG-3′
T-Tub2	5′-GGT CCT CGG AGT GGA GAG GG-3′
cpc-Eco	5′-CAA GAA TTC GCC AAG ATG-3′
cpc-Sal	5′-CGA AAT CCC GTG TCG ACA AAA G-3′
mbf-Eco	5′-CCC GAA TTC AAA ATG GAT GAC TGG-3′
mbf-Sal	5′-GCA GTC GAC GCA ATT TCT CC-3′
cpc_SOSfor(Nco)	5′-GGA TCC ATG GCT AGC AGA GAA TTC GCC-3′
cpc_SOSrev(Sal)	5′-GCT CTA GAG TCG ACT AAT ACT CTC GAG-3′
mbf-Kpn	5′-CGT CAG GGT ACC GCT TCA TC-3′
mbf-Sal-ko	5′-GGT GGT ATC CCA GTC GAC C-3′
mbf-Hind	5′-CCC AAG TTC CCC AAC AAG CTT AA-3′
mbf-Bam	5′-CCT TGA CTA AAG GAC GGA TCC-3′
T-Tub-for-Hind	5′-GAG AAG CTT TTC ATC GGT CTC AAG TCC CG-3′
T-Tub-rev-Xho-Hind	5′-CCA TCT ATA TAA GCT TTC TCG AGC AGC TGC C-3′
ccg1-P-Sal	5′-GGA GCA GTC GAC CTG CGT GAA TCA CG-3′
OliC-for-Xho	5′-GCC GCA TTC TCG AGT CGG GCC GGA TTG GTC-3′
hph-rev-Hind	5′-CGG CGA AAG CTT CTA CAC AGC CAT CGG-3′
GFP-Eco-Sph	5′-TTA CTT GAA TTC CTC GTG CAT GCC-3′
cpc1-GFP-for	5′-CAG GAA GTG GCA TGC AAC AAA CCG CCA-3′
cpc1-GFP-rev	5′-CCA CTT TTG AAT TCC ATT ATT GCA GTC C-3′
cpc-RACE-1	5′-TTC GTT GGC GCT CAT AAC CCC AGC CG-3′
cpc-RACE-2	5′-GCA CCG GGA AAG CCA GCA GGA CTA CG-3′

library of *F. fujikuroi* m567 (Malonek, unpubl.) were plated with *E. coli* strain XL1-blue MRF' (Stratagene, La Jolla, CA) and screened by plaque hybridization (Sambrook et al., 1989) with a [^{32}P]dCTP-labelled 0.6-kb PCR fragment of the *F. fujikuroi cpc1* gene and the cDNA fragment of the *F. fujikuroi mbf1* gene, respectively, as probes.

2.4. Macroarray screening

RNA isolation, cDNA library construction and macroarray screening was performed as described (Teichert et al., 2004). The macroarrays were differentially hybridized with cDNA probes from the wild-type and from the Δ*cpc1* strain cultivated in ICI medium with addition of 4 mM MSX as described above. Quantitative analyses of spot intensity and expression comparison was carried out by Arrayvision™ (Imaging Research Inc., St. Catherines, Canada) and using the FiRE® macro collection FiRe (Garcion et al., 2006). The experiment was done with two different cultures and repeated four times for each probe. Clones which showed at least twofold up-regulation or down-regulation in comparison to the wild-type were chosen for further analysis.

2.5. Nucleic acid isolation, Southern and northern blot analysis

Genomic DNA was isolated as described previously (Cenis, 1992). Lambda DNA was isolated as described (Sambrook et al., 1989). Plasmid DNA was extracted using a plasmid extraction kit (Qiagen, Hilden, Germany). For Southern-blot analysis, genomic, plasmid or phage DNA was restricted with appropriate restriction enzymes, fractionated in 1.0% (w/v) agarose gels, and transferred to nylon membranes (N^+, GE Healthcare, Little Chalfont, UK) by downward blotting. Hybridization was carried out in hybridization buffer (6x SSC, 5x Denhardt's solution and 0.1% SDS, and 50 mM phosphate buffer, pH 6.6) at 65 °C. Membranes were washed once (2x SSPE, 0.1% SDS) before being exposed to autoradiographic film. DNA probes were labelled using a random prime labelling kit (GE Healthcare).

Total *F. fujikuroi* RNA was isolated using the RNAgents Total RNA isolation kit (Promega, Mannheim, Germany). For northern blot analysis, 20 μg total RNA was transferred to Hybond-N + membranes after electrophoresis on a 1% agarose gel containing formaldehyde, according to Sambrook et al. (1989). Blot hybridizations were carried out in 0.6 M NaCl, 0.16 M Na_2HPO_4, 0.06 M EDTA, 1% N-lauroylsarcosine (Sigma–Aldrich, St Louis, MO, USA), 10% dextran sulphate (Eppendorf AG, Hamburg, Germany), 0.01% salmon sperm DNA, pH 6.2, at 65 °C in the presence of a random-primed [α-^{32}P]-dCTP labelled probe. Membranes were washed in 2x SSPE, 0.1% SDS at 65 °C before being exposed to autoradiographic film.

2.6. PCR, RT-PCR and RLM-RACE PCR

PCR reactions contained 25 ng DNA, 50 ng of each primer, 0.2 mM dNTPs and 2 U Biotherm polymerase (Genecraft, Lüdinghausen, Germany) in 50 μl. PCR was carried out at 94 °C for 4 min followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 70 °C for 1.5 min using the primer listed in Table 1. cDNA templates were prepared using the SUPERSCRIPT™ One-Step RT-PCR with PLATINUM Taq Kit (Invitrogen, Groningen, The Netherlands). The TAIL-PCR was performed following as described (Terauchi and Kahl, 2000). Specific PCR reaction steps were carried out at 60 °C. Oligonucleotides used (cpc-T1, cpc-T2, cpc-T3, cpc-T4, cpc-T5 and RAPD-E4) are listed in Table 1.

RLM-RACE PCR was conducted using Ambion's FirstChoice® RLM-RACE kit according to the manufacturer's instructions. cDNA was synthesized using the SUPERSCRIPT™ One-Step RT-PCR with PLATINUM Taq Kit (Invitrogen, Groningen, The Netherlands) with a poly-T primer. Specific nested primers for amplification of the *cpc1* 5′ untranslated region were cpc-RACE-1 for the inner and cpc-RACE-2 (see Table 1) for the outer PCR.

2.7. Fungal transformations

Preparation of protoplasts and transformations were carried out as described (Tudzynski et al., 1996). 10^8 protoplasts of strains IMI58289 were transformed with 10 μg of the *Sac*I/*Sal*I fragment of the replacement vector pΔcpc1, or 15 μg of the circular plasmid pcpc1-Hind2.7 for complementation of the Δ*cpc1* mutant, or 15 μg of the circular vector pWMS-cpc1 to obtain mutants expressing GFP-Cpc1 fusion proteins. Transformed protoplasts were regenerated at 28 °C in complete regeneration agar (0.7 M sucrose, 0.05% yeast extract, 0.1% casamino acids) and 120 μg ml^{-1} nourseothricin or 100 μg ml^{-1} hygromycin, respectively, for 6–7 days.

2.8. Yeast two-hybrid analyses

Yeast two-hybrid analyses were performed following the manufacturer's instructions using the CytoTrap® XR Library Construction Kit (Stratagene, La Jolla, USA) and the Hybri-ZAP®-2.1 Two-Hybrid kit (Stratagene, La Jolla, USA). Hybri-ZAP®-2.1 two-hybrid experiments were performed with the *S. cerevisiae* SMY3 strain (Cardenas et al., 1994) as recipient strain to avoid the reddish colour of the Y190 strain included in the kit. For the Cytotrap® two-hybrid, the bait protein is fused to a myristoylation signal, which targets it to the cell membrane, whereas the target protein is fused

to the hSOS protein, a homologue of the *S. cerevisiae* CDC25 protein, a guanyl exchange factor that activates the RAS signalling cascade. The *S. cerevisiae* recipient strain CDC25H harbours a temperature sensitive mutation in the CDC25 protein, which prevents growth at 37 °C. If the two proteins of interest interact, the hSOS moiety is brought to the plasma membrane, where it can activate the membrane-localized RAS and the RAS signalling cascade, thus re-establishing growth at 37 °C.

2.9. Cytological techniques

For microscopy, strains were cultivated on agar coated glass slides (Diagonal, Münster, Germany). Nuclei were stained with Hoechst 33342 (Sigma) (15 mg ml^{-1} in 1 ml McIlvaine buffer, pH 7.2). Epifluorescence was observed using a Leica DMRBE microscope (Leica, Wetzlar, Germany) with a high performance charge-coupled device (CCD)-camera (12 bit SensiCam; PCO AG, Kelheim, Germany) and filter set A (excitation filter 340–380, dichromatic mirror 400, long pass filter 425) for observation of the Hoechst nuclei staining or filter set L5 (excitation filter BP 480/40, dichromatic mirror 505, suppression filter BP 527/30) for GFP fluorescence.

2.10. Nucleotide accession numbers

The *cpc1* sequence was submitted to GenBank under accession number AM906209, and the *mbf1* sequence under the accession number AJ698905. Sequences of the Cpc1 target genes were deposited at GenBank under the accession numbers specified in Table 2.

3. Results

3.1. Cloning and gene replacement of cpc1

The genomic copy of *cpc1* was cloned by PCR using primers derived from the nucleotide sequence of the *Fusarium graminearum* homologue (FG09286.1) and subsequent TAIL-PCR (Terauchi and Kahl, 2000) and gene library screening (see Section 2 for further details). The *cpc1* ORF has a length of 702 bp, interrupted by an intron of 51 bp. The resulting putative protein of 233 amino acids shares 98% amino acid sequence identity with *Fusarium verticillioides* FVEG_03822.3, 80% with *F. graminearum* FG09826.1, 55% with *N. crassa* Cpc1 (CAE85619), 34% with *A. nidulans* CpcA (AAL09315) and 26% with *S. cerevisiae* GCN4 (AAL09032). RLM-RACE-PCR analysis and examination of 26 EST clones of *cpc1* of a *F. fujikuroi* cDNA library (Teichert et al., 2004) revealed only one transcriptional start site at 686 bp upstream of the translation start whereas the poly-A tail is added 872 bp downstream of the start codon, resulting in a transcript size of 1507 bp after the excision of the 51-bp intron.

The *cpc1* orthologues of *S. cerevisiae*, and those of *A. nidulans* and *N. crassa* are preceded by two or four uORFs, respectively (Hinnebusch, 1984; Hoffmann et al., 2001; Paluh et al., 1988). In yeast, these uORFs are required for the translational regulation of *gcn4* by attenuating efficient translation of the ORF itself under sufficient amino acid supply (Hinnebusch, 1997). The same mechanism applies to *cpc-1* in *N. crassa* (Sattlegger et al., 1998) and *cpcA* in *A. nidulans* (Sasse et al., 2008). In *F. fujikuroi*, two possible uORFs of a length of 2 and 34 amino acids were identified in the *cpc1* promoter region (at positions −607 and −385), with the second uORF sharing 54% amino acid identity with the *N. crassa* second uORF. Like *N. crassa* (Paluh et al., 1988), *F. fujikuroi* seems to possess only one binding site for the *cpc1* gene product itself (Cpc1 response element, CPRE) in its promoter region, matching exactly the consensus sequence of 5′-TGACTCA-3′ which was recently identified in numerous promoters of Cpc1 target genes of *N. crassa* (Tian et al., 2007). The genomic organization of *cpc1* is presented in Fig. S1.

To further characterize the function of Cpc1 in *F. fujikuroi*, the gene was deleted by targeted gene replacement (see Section 2). As all deletion mutants showed a similar phenotype, transformant Δ*cpc1*-T26-2 was selected for further analysis.

3.2. Cpc1 is not responsible for down-regulation of gibberellic acid biosynthesis genes in the ΔglnA mutant

To analyze if the unexpected down-regulation of genes involved in GA and bikaverin biosynthesis in the Δ*glnA* mutant was due to the action of Cpc1 as it has been suggested for penicillin biosynthesis genes in *A. nidulans* (Busch et al., 2003), we incubated both the wild-type and the Δ*cpc1* mutant in media containing either no nitrogen, 100 mM glutamine or no nitrogen and 4 mM L-methionine-D,L-sulfoximine (MSX), which binds irreversibly to the

Table 2
Target genes of Cpc1 identified in macroarray analyses.

Name	Accession #	Closest homolog	e-Value	Fold change Δ*cpc1*/WT[a]	Frequence
AA biosynthesis					
leu4	FN297827	2-Isopropylmalate synthase [*Neurospora crassa*]	1e−34	0.21	1
leu2	FN297826	3-Isopropylmalate dehydrogenase [*Saccharomyces cerevisiae*]	1e−105	0.43	2
lys1	FN297828	Saccharopine dehydrogenase (NAD+, L-lysine forming) [*Aspergillus fumigatus*]	1e−100	0.34	3
lys21	FN297830	Homocitrate synthase [*S. cerevisiae*]	4e−134	0.23	2
lys9	FN297829	Saccharopine dehydrogenase ($NADP^+$, L-glutamate forming) [*S. cerevisiae*]	1e−117	0.48	1
hom2	FN297823	Aspartate-semialdehyde dehydrogenase [*A. fumigatus*]	1e−102	0.27	1
cpa1	FN297821	Carbamoyl-phosphate synthase, small chain [*Gibberella zeae*]	5e−67	0.35	3
arg6	FN297820	Mitochondrial precursor (contains: N-acetyl-glutamate semialdehyde dehydrogenase, N-acetyl-L-glutamate 5- phosphotransferase) [*N. crassa*]	0.0	0.48	1
aat1	FN297819	Aspartate aminotransferase [*N. crassa*]	0.0	0.38	1
his3	FN297822	Histidine biosynthesis trifunctional protein (includes: phosphoribosyl-AMP cyclohydrolase; phosphoribosyl-ATP pyrophosphohydrolase; histidinol dehydrogenase) [*N. crassa*]	1e−81	0.40	1
ilv5	FN297825	Acetohydroxy-acid isomeroreductase, mitochondrial precurser [*N. crassa*]	6e−94	0.40	2
Other					
hat	CAG28690.1	Putative histone acetyltransferase [*Fusarium fujikuroi*]	1e−93	0.20	2
idh	FN297824	Isocitrate dehydrogenase, NAD-dependent [*A. clavatus*]	6e−63	0.24	1
mbf1	AJ698905	Multi-protein bridging factor [*F. fujikuroi*]	2e−79	0.29	2
pre5	FN297831	Proteasome alpha type-1 component [*A. fumigatus*]	1e−103	0.34	2

[a] "Fold change" represents the mean expression value of the respective gene in the *cpc1* mutant divided by the value of the gene in the wild-type. Genes with "fold changes" $\leqslant 0.5$ or $\geqslant 2$ are regarded as differentially regulated under the specific conditions. If genes were identified several times in the macroarray analyses ("frequence" >1), their fold change values were averaged.

catalytic core of the glutamine synthetase octamer (Manning et al., 1969). Total RNA was extracted and submitted to northern blot analysis (Fig. 1).

As described before (Teichert et al., 2004), the GA biosynthetic gene *cps/ks* (Tudzynski, 2005) and the bikaverin biosynthetic gene *bik3* (Wiemann et al., 2009) are highly expressed in the absence of nitrogen, but repressed either upon addition of glutamine or the specific GS inhibitor MSX. The expression of both genes is similar in the Δ*cpc1* mutant compared to the wild-type, also when MSX is added, making it unlikely that Cpc1 is responsible for the down-regulation of GA and bikaverin genes when the GS is inhibited by MSX, or in the Δ*glnA* mutant.

3.3. Identification of Cpc1 target genes

In order to identify target genes of Cpc1, macroarray experiments were performed where the expression profile of the wild-type was compared to that of the Δ*cpc1* mutant, both treated with MSX to induce starvation for the amino acid glutamine. Hybridization of the macroarrays was performed three times with two independent biological runs as described (Teichert et al., 2004). Target genes were identified using the program Arrayvision™ (Imaging Research Inc.) and confirmed by subsequent northern blot analysis (Table 2, Fig. 2).

Among the target genes identified were those involved in biosynthesis of several amino acids, e.g. of leucine (*leu2* and *leu4*), lysine (*lys1*, *lys9* and *lys21*), aspartic acid (*hom2*), arginine (*cpa1* and *arg6*), aspartate and threonine via homoserine (*aat1*), histidine (*his3*), and of isoleucine, leucine and valine (*ilv5*) (see Table 2 for details).

Furthermore, several other genes not involved in amino acid biosynthesis were found to be up-regulated in the *cpc1* mutant, e.g. *idh* encoding the isocitrate dehydrogenase responsible for the formation of 2-oxoglutarate in the TCA cycle, and *pre5* coding for the α subunit of the 20S proteasome. Additionally, a gene coding for a putative acetyltransferase was shown to be down-regulated in the *cpc1* mutant. This gene had also been identified as GS target gene that is up-regulated in the *glnA* knock-out mutant (Teichert et al., 2004). Another Cpc1 target gene had also previously been found to be up-regulated in the Δ*glnA* mutant: *mbf1*, coding for a potential homologue of the co-activator of GCN4 in *S. cerevisiae* (Takemaru et al., 1998).

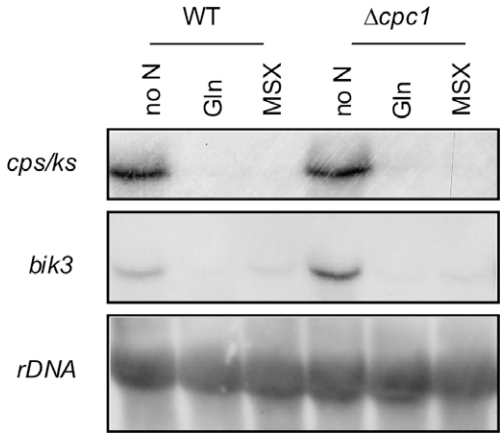

Fig. 1. Cpc1 does not repress secondary metabolite genes. No N: no nitrogen source present in the media; Gln: addition of 100 mM glutamine; MSX: addition of 4 mM MSX to a medium containing no nitrogen source. The filter was hybridized with a radioactively labelled rDNA probe to assure uniform loading of the gel.

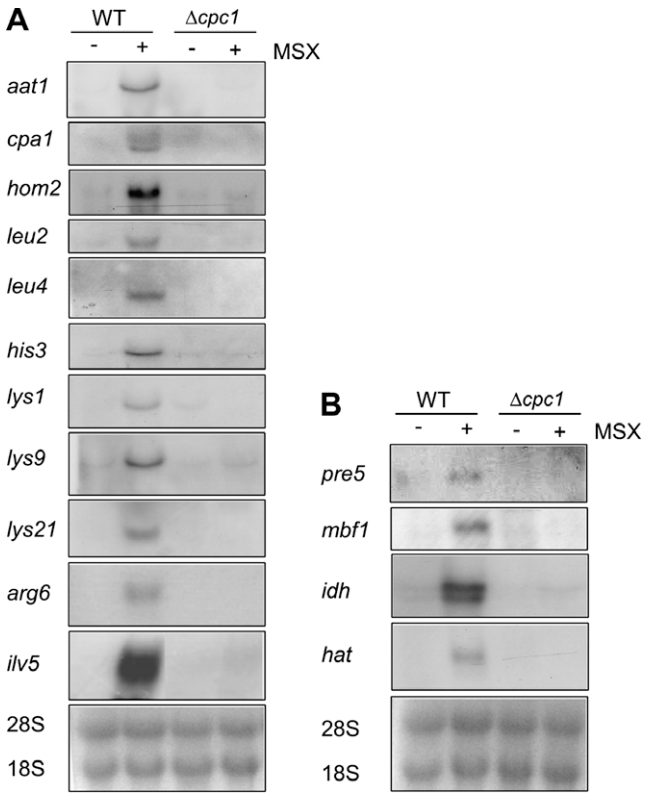

Fig. 2. Verification of Cpc1 target gene expression. (A) Amino acid biosynthesis genes. (B) Genes not implicated in amino acid biosynthesis. Wild-type (WT) and *cpc1* deletion mutant (Δ*cpc1*) were grown under nitrogen starvation conditions. Where indicated by a plus sign, MSX was added to a final concentration of 4 mM. 28S and 18S rRNA is shown as a loading control. Probes are listed in Table 2.

3.4. Cloning and gene replacement of mbf1

In *S. cerevisiae*, the Cpc1 homologue GCN4 requires the multiprotein bridging factor MBF1 to recruit the TATA-box binding protein to GCN4, bound to the promoter of its target genes (Takemaru et al., 1998). To see if there was a functional connection between Cpc1 and its potential co-activator Mbf1 also in *F. fujikuroi*, the *mbf1* gene was isolated in a genomic lambda library screening. The *mbf1* ORF has a length of 459 bp and is interrupted by a 48-bp intron. The predicted Mbf1 protein has a length of 152 amino acids, with 62% and 63% amino acid identity to the putative homologues in *N. crassa* (XP_960690) and *A. nidulans* (XP_660600.1), respectively, and 49% to *S. cerevisiae* MBF1 (NP_014942.2). In its promoter region, one putative CPRE was found at position −497. To test if Mbf1 is implicated in cross-pathway control in *F. fujikuroi*, Δ*mbf1* deletion mutants were generated (see Section 2). As all mutant strains showed a similar phenotype, strain Δ*mbf1*-T27-3-2 was selected for further analysis.

3.5. The phenotype of Δcpc1 and Δmbf1 mutants

To analyze if Mbf1 and Cpc1 were functionally interconnected, we compared the growth of Δ*cpc1* and Δ*mbf1* mutants on media with different nitrogen sources and with or without MSX. Mutants lacking a functional *cpc1* gene are expected to be unable to up-regulate amino acid biosynthesis. Thus, inhibitors of amino acid biosynthesis such as MSX would have a stronger inhibitory effect in these mutants because they cannot compensate the inhibition of the respective enzymes by transcriptional up-regulation. Additionally, growth of wild-type and Δ*cpc1* and Δ*mbf1* mutants was studied on media containing homoserine or isoleucine as nitrogen

source (Fig. 3). The *N. crassa* Δ*cpc-1* mutant had been reported to be unable to grow on media containing these amino acids, since the accumulation of large internal pools of the effective amino acid might inhibit common steps of the branched pathway of amino acid biosynthesis (Barthelmess, 1986). Furthermore, the response of the wild-type and both mutants to H_2O_2 was tested, since the *S. cerevisiae* GCN4 protein is involved in the response to reactive oxygen species (Mascarenhas et al., 2008).

The addition of MSX strongly impeded growth of Δ*cpc1* (Fig. 3), whereas addition of H_2O_2 had no effect. Moreover, growth of Δ*cpc1* on L-homoserine and L-isoleucine as only nitrogen source was significantly reduced. In contrast, the phenotype of the Δ*mbf1* mutant is comparable to that of the wild-type on all media (Fig. 3).

To examine if the phenotype of the *cpc1* deletion mutant was rescued by complementation with the wild-type *cpc1* gene, Δ*cpc1*-T26-2 was transformed with vector pcpc1-Hind2.7 carrying the entire *cpc1* ORF as well as 1.3 kb of the 5′ UTR and 0.5 kb of the 3′ UTR. Transformants growing on regeneration medium containing 4 mM MSX were checked for integration of the *cpc1*wild-type gene copy and presence of the *cpc1* replacement cassette (data not shown). The complementation mutant *cpc1*^{comp}-T10-1 shows a wild-type-like phenotype under all conditions demonstrating that the deletion of *cpc1* is responsible for altered growth pattern (Fig. 3).

3.6. Interaction studies of Mbf1 and Cpc1

To analyze if the *F. fujikuroi* Cpc1 and Mbf1 proteins interacted with each other as described for the *S. cerevisiae* homologues (Takemaru et al., 1998), a yeast two-hybrid experiment was set up. The plasmids pBD-Mbf and pAD-Cpc1 (see Section 2) were transformed into *S. cerevisiae* strain SMY3, and the transcriptional activation of the reporter genes *HIS3* and *lacZ* was tested as indication for a possible interaction between Cpc1 and Mbf1. As none of the *S. cerevisiae* strains carrying both the pBD-Mbf and the pAD-Cpc1 vectors were able to grow on medium lacking histidine or showed a blue colour in a X-Gal filter lift experiment (Fig. S2A), AD-Cpc1 and BD-Mbf1 proteins do not interact in this assay. For this reason, an alternative yeast two-hybrid approach, the Cyto-Trap® system (see Section 2) was used to analyze a possible interaction of Cpc1 and Mbf1. Again, Cpc1/Mbf1 interaction was not detected, as all four pSOS-Cpc1/pMyr-Mbf1 co-transformants failed to grow at 37 °C (Fig. S2B). Since in this system there always is the possibility that transcription factors are targeted to the nucleus due to their nuclear localization signal, correct localization of the Cpc1 protein in the cytoplasm was confirmed by co-transformation of pSOS-Cpc1 with vector pMyr-SB (Stratagene, La Jolla, CA), which contains a SOS binding protein targeted to the cell membrane by the myristoylation signal. If the SOS fusion protein is freely accessible in the cytoplasm, the SOS binding protein binds the hSOS moiety and the RAS cascade is activated. As seen in Fig. S2B, pSOS-Cpc1/pMyr-SB co-transformants grew comparably to the positive control, indicating that the absence of growing transformants in the pSOS-Cpc1/pMyr-Mbf1 transformants is not due to faulty localization of the hSOS-Cpc1 fusion protein.

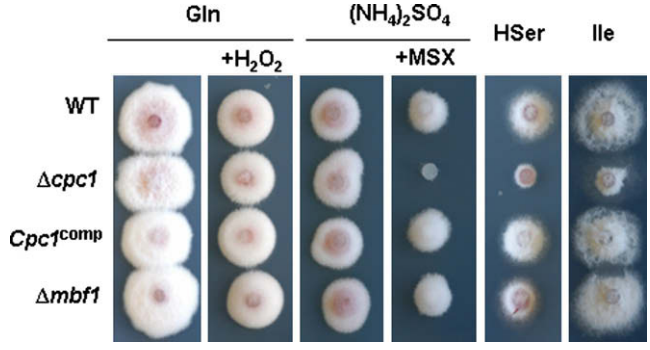

Fig. 3. The Δ*cpc-1* mutant is more susceptible to inhibitors of amino acid biosynthesis. Agar plates containing 100 mM L-glutamine, 10 mM $(NH_4)_2SO_4$, 100 mM L-homoserine (HSer) or 100 mM L-isoleucine (Ile) were inoculated with agar plugs of defined size of the wild-type, the *cpc1* deletion mutant T26-2 (Δ*cpc1*), the Δ*cpc1* complementation mutant T10-1 (*cpc1*^{comp}) and the *mbf1* deletion mutant (Δ*mbf1*). 3-amino triazole (3-AT), L-methionine-D, L-sulfoximine (MSX) and 30 mM H_2O_2 were added to the medium containing the nitrogen source specified.

3.7. Regulation of cpc1 expression

To test if AreA and GS, which are both involved in the nitrogen regulation network, were related to cross-pathway control and if Mbf1 was involved in this network, we studied the expression of *cpc1* and several AreA and Cpc1 target genes in the wild-type and the Δ*cpc1*, Δ*mbf1*, Δ*areA*, and Δ*glnA* deletion mutants. The strains were pre-cultivated in ICI medium and washed mycelia were transferred into media containing either no nitrogen, no nitrogen and MSX, or glutamine, respectively (Fig. 4).

In the wild-type, *cpc1* is only weakly expressed in the absence of nitrogen. Expression increases significantly upon addition of glutamine, and even more upon addition of the GS inhibitor MSX. In the Δ*mbf1* mutant, the expression of *cpc1* is slightly up-regulated under nitrogen starvation conditions and less up-regulated upon MSX addition compared to the wild-type, suggesting that Mbf1 has at least some role in regulation of *cpc1* transcription. Surprisingly, expression of *cpc1* is significantly up-regulated under nitrogen starvation in both the Δ*areA* and the Δ*glnA* deletion mutants compared to the wild-type. The expression of *mbf1* is also clearly increased in the Δ*areA* and Δ*glnA* mutants under nitrogen starvation conditions as shown previously (Schönig et al., 2008; Teichert et al., 2004), but almost completely abolished in the Δ*cpc1* mutant as expected for *mbf1* being a Cpc1 target gene. The expression of two other Cpc1 target genes, *leu4* and *hat*, is similar to the *mbf1* expression, and not significantly reduced in Δ*mbf1*. The expression pattern of the *glnA* gene itself is not affected in the

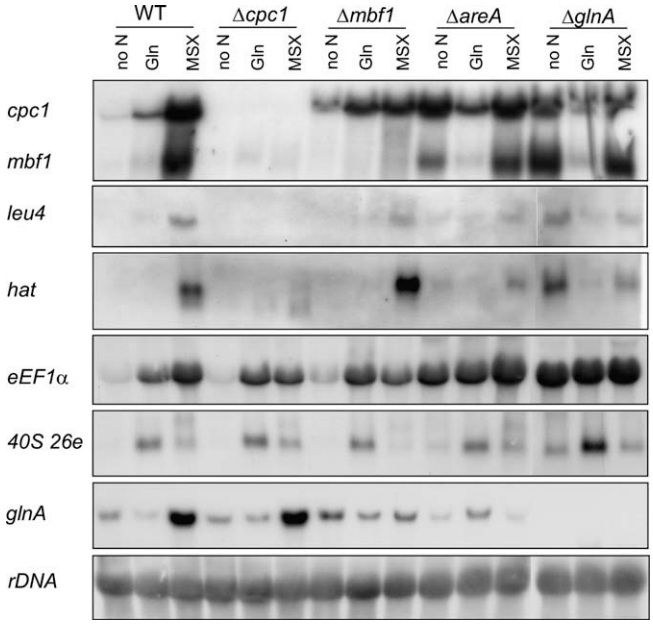

Fig. 4. The deletion of *mbf1* has no effect on Cpc1 and GS target gene expression. The wild-type and the deletion mutants Δ*cpc1*, Δ*mbf1*, Δ*areA* and Δ*glnA* were grown in the following media: No N: no nitrogen source present in the media. Gln: addition of 100 mM glutamine; MSX: addition of 4 mM MSX to a medium containing no nitrogen source. The filter was hybridized with a radioactively labelled rDNA probe to assure uniform loading of the gel.

Δ*cpc1* mutant, although it encodes a central amino acid biosynthetic gene, underlining the special role of GS compared to other amino acid biosynthetic genes. The expression of the GS-regulated genes *eEF1α* and *40S 26e* (Teichert et al., 2004), coding for a translation elongation factor and a 40S ribosomal protein subunit was neither significantly altered in the Δ*cpc1* nor in the Δ*mbf1* mutant, but significantly up-regulated in the Δ*glnA* and Δ*areA* mutants (Fig. 4).

To study the effect of different nitrogen sources and amino acids on the expression of *cpc1* and its target genes, the wild-type was shifted into media containing either no nitrogen, a dropout mixture (DO, Clontech) of all amino acids, varying concentrations of different nitrogen sources or several toxic compounds affecting nitrogen or amino acid metabolism, such as MSX, the TOR kinase inhibitor rapamycin, and 3-AT. 3-AT is a false feedback inhibitor of the imidazoleglycerol phosphate dehydratase (Klopotowski and Wiater, 1965), a key enzyme of histidine biosynthesis. Total RNA was extracted and used for northern blot analysis (Fig. 5).

Again, *cpc1* expression is very weak under nitrogen starvation conditions, and increases upon addition of any nitrogen source. While the addition of a commercially available amino acid dropout mixture (DO) and the addition of a limited amount of $(NH_4)_2SO_4$ leads to an increase in *cpc1* transcription, larger concentrations of $(NH_4)_2SO_4$ or amino acids like asparagine and glutamine even stronger augmented *cpc1* expression. A similarly marked up-regulation of *cpc1* can be observed upon the addition of 3-AT, and even more upon addition of MSX to nitrogen-starved mycelia, whereas addition of the TOR kinase inhibitor rapamycin does not affect *cpc1* transcription. The expression of Cpc1 target genes, such as *lys21* and *ilv5*, follows in general the expression of *cpc1* itself, while *glnA* is clearly induced upon DO, rapamycin or MSX addition. The expression of *mbf1* is up-regulated by DO and MSX. As expected, the GA biosynthesis gene *cps/ks* was only expressed under nitrogen

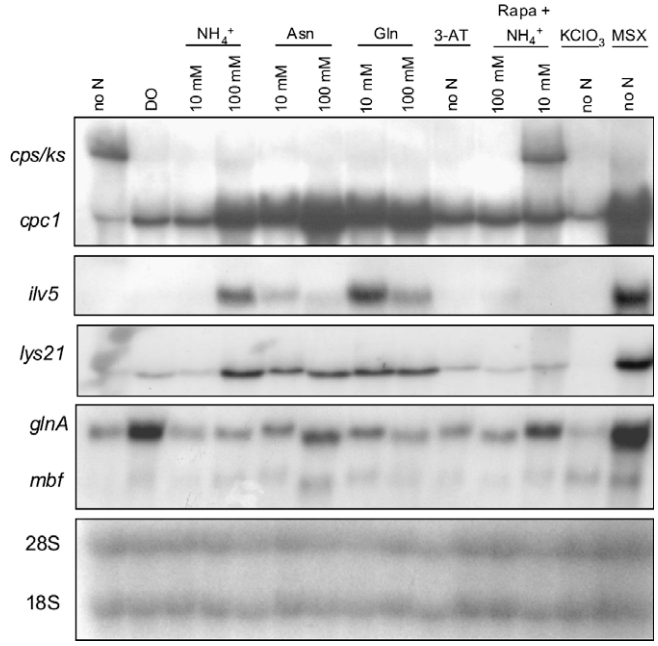

Fig. 5. *Cpc1* is differentially expressed dependent on varying nitrogen conditions. DO: addition of a commercially available amino acid mixture (1x Clontech –ade/-his/-leu/-trp DO supplement), with 20 mg l^{-1} L-adenine hemisulfate, 20 mg l^{-1} L-histidine HCl monohydrate, 100 mg l^{-1} L-leucine and 20 mg l^{-1} L-tryptophan added after autoclaving; NH_4^+: 10 or 100 mM $(NH_4)_2SO_4$ added; Asn: 10 or 100 mM L-asparagine added. 3-AT: 40 mM 3-AT added to medium without nitrogen; Rapa: 200 ng ml^{-1} rapamycin added to medium containing 10 mM $(NH_4)_2SO_4$; $KClO_3$: 60 g l^{-1} $KClO_3$ added to medium containing no nitrogen source; MSX: 4 mM MSX added to medium containing no nitrogen source. 28S rRNA is shown as a loading control.

starvation conditions, or upon addition of rapamycin to a medium with 10 mM $(NH_4)_2SO_4$ (Fig. 5).

The deletion of the *glnA* gene had led to a significant up-regulation of *cpc1* and down-regulation of GA biosynthetic genes under nitrogen starvation conditions (Teichert et al., 2004). To examine if these effects are a general feature of mutants auxotrophic for one of the 20 proteinogenic amino acids, we analyzed the expression of *cpc1*, the GA gene *cps/ks*, *glnA* and several Cpc1 target genes in a glutamate auxotrophic mutant which had been generated by double knock-out of the two glutamate biosynthetic genes *gltA* and *gdhA*, encoding the glutamate synthetase and the NADP(+)-dependent glutamate dehydrogenase (Huber and Tudzynski, unpublished). We also studied expression of these genes in randomly generated UV mutants auxotrophic for histidine, lysine, leucine and methionine. All strains were grown for 4 days in synthetic ICI medium supplemented with the strain-specific missing amino acid and then transferred into nitrogen free medium to induce starvation. After 4 h, either glutamine, a DO mixture, the complementing amino acid, or water was added to the flasks, and the cultures were incubated for two more hours. Total RNA was extracted and used in a northern blot experiment (Fig. 6).

Transcription of *cpc1* is very low in the wild-type under nitrogen starvation, and slightly increased in the auxotrophic mutants under the same conditions. The addition of glutamine or the specific amino acid strongly enhances *cpc1* transcription in the auxotrophic mutants, while the addition of DO mixture does not elevate *cpc1* transcription above the level observed in the wild-type. Interestingly, the up-regulation of *cpc1* under the described conditions results directly in up-regulation of Cpc1 target genes, such as *his3*, *lys1*, *hom2*, *leu2* and *mbf1*. The expression of *glnA* is different: the gene is not a target of Cpc1 and shows a high expression level under most growth conditions, and even greater expression after DO addition.

Interestingly, the GA gene *cps/ks* is expressed in all auxotrophic mutants under nitrogen starvation conditions in contrast to the *glnA* deletion mutant, demonstrating again the specific role of GS in the nitrogen regulation network. Addition of glutamine or the complementing amino acid, and to a lesser extent, of the DO mixture, leads to the repression of *cps/ks*. Only in the methionine mutant, the repression of *cps/ks* by nitrogen sources is weaker compared to the wild-type (Fig. 6).

3.8. Cpc1 localization depends on the nitrogen status of the cell

To study the intracellular localization of Cpc1 in dependence on the nitrogen status, a *cpc1*::*gfp* fusion construct was generated (for details see Section 2) and transformed into the Δ*cpc1* mutant. The transformants were selected for hygromycin resistance and growth on medium containing 2 mM MSX indicating the restoration of Cpc1 function (data not shown).

The localization of the fusion protein was analyzed under differing nitrogen conditions and with and without MSX. Epifluorescence microscopy of a transformant incubated under nitrogen starvation conditions or on a medium containing glutamate as nitrogen source, either with or without 2 mM MSX, showed that the Cpc1-GFP fusion protein was localized predominantly in the cytoplasm under nitrogen starvation conditions and translocated to the nucleus upon MSX addition (Fig. 7). Under conditions of abundant nitrogen, Cpc1 is located in the nucleus regardless of MSX addition.

4. Discussion

Previously, we have shown that the deletion of the glutamine synthetase gene *glnA* leads to a total down-regulation of GA

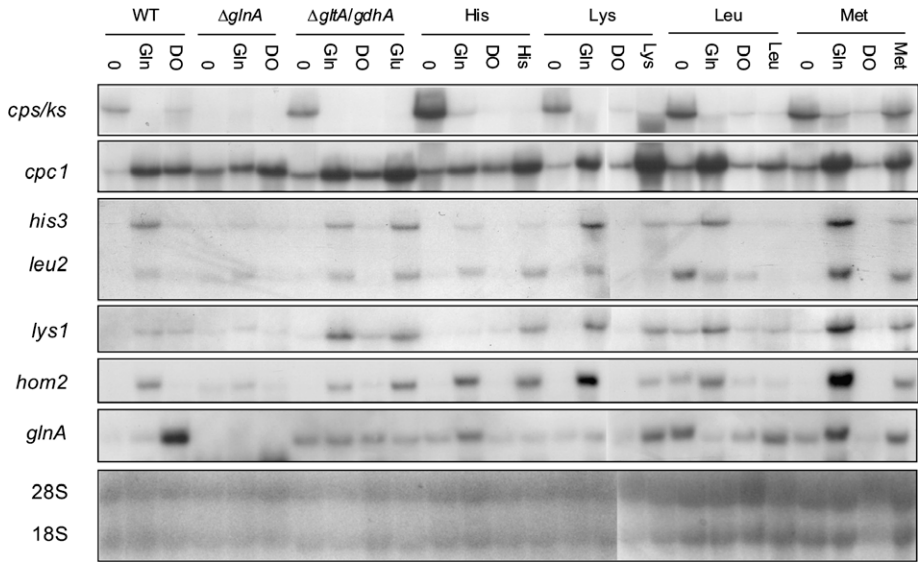

Fig. 6. Deregulation of the cross-pathway control in amino acid auxotrophic mutants. The wild-type (WT) and glutamine (Δ*glnA*), glutamate (Δ*gltA*/*gdhA*), histidine (His), lysine (Lys), leucine (Leu) and methionine (Met) auxotrophic mutants were incubated in the presence of either no nitrogen (0), 100 mM glutamine (Gln), or a commercially available dropout mixture of all proteinogenic amino acids (DO, Clontech), supplemented with the lacking amino acids, or additionally with 100 mM of glutamate (Glu), histidine (His), lysine (Lys), leucine (Leu), methionine (Met) for the respective auxotrophic mutants. 28S and 18S rRNA is shown as a loading control.

biosynthesis and up-regulation of *cpc1* under conditions of nitrogen starvation. In order to examine whether Cpc1 is involved in this unexpected repression, and to study the role of Cpc1 in *F. fujikuroi* in detail, we cloned and characterized the *cpc1* gene.

4.1. The role of Cpc1 is restricted to up-regulation of amino acid biosynthesis in response to amino acid imbalances

The deletion of *cpc1* led to an increased susceptibility to toxic amino acid analogues, such as 3-AT and MSX, due to the inability of the mutant to compensate for the induced amino acid starvation by increased transcription of genes involved in amino acid biosynthesis. In contrast to the *S. cerevisiae gcn4* mutant, the *cpc1* mutant showed a wild-type-like phenotype during growth under oxidative stress induced by H_2O_2 (Fig. 3). These results indicate that Cpc1 plays a specific role only in amino acid stress response in *F. fujikuroi*. This conclusion is also supported by the predicted functions of the target genes of Cpc1 in *F. fujikuroi* identified by macroarray analyses: Most of them are involved in amino acid biosynthesis.

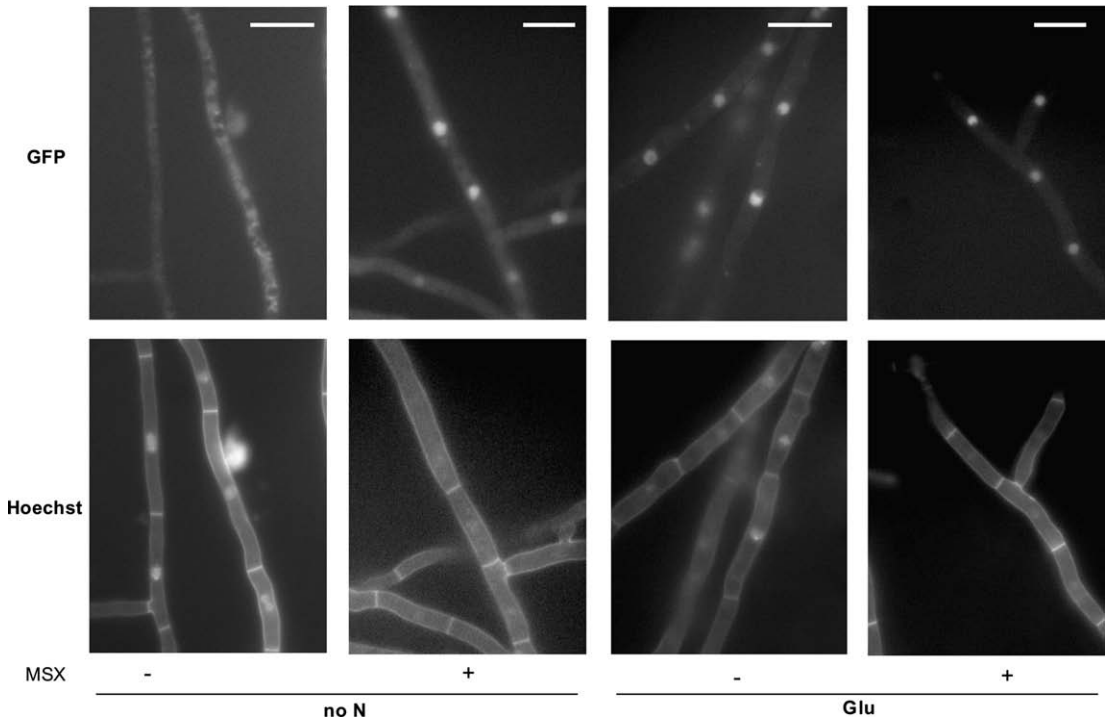

Fig. 7. Cpc1 localization depends on the nitrogen status of the cell. The Cpc1-GFP strain was grown on ICI media containing either no nitrogen source (no N) or 10 mM glutamate (Glu). Where indicated, MSX was added to a final concentration of 2 mM. The bars represent a length of 10 μm. GFP: GFP epifluorescence. Hoechst stain: staining of cell wall, septae and nuclei.

Recently, 443 CPC1 target genes had been identified in *N. crassa* (Tian et al., 2007), among them also homologues to the genes *hom2*, *lys1*, *arg6*, *his3*, *leu2*, *leu4*, *lys21* and *cpa1* found to be Cpc1 target genes in *F. fujikuroi*. Three other Cpc1 target genes might have an indirect influence on amino acid biosynthesis. Thus, the isocitrate dehydrogenase (*idh*) is responsible for formation of 2-oxoglutarate, the precursor of glutamate and various other amino acids, while the 20S proteasome α subunit (*pre5*) might help to re-fill the amino acid pool of the cell under amino acid starvation by degradation of proteins. The category of genes involved in protein degradation was also enriched in the *S. cerevisiae* and the *Candida albicans* datasets of GCN4 and CaGCN4 target genes (Tian et al., 2007). The third gene, *hat*, is not involved in amino acid biosynthesis but had already been found to be up-regulated in the *glnA* deletion mutant (Teichert et al., 2004).

The smaller number of Cpc1 target genes compared to the dataset of the *N. crassa* Cpc1 and *S. cerevisiae* GCN4 target genes is likely due to the fact that the macroarray used comprises about 3000 different ESTs, which represent only a small fraction of the estimated number of 14000 *F. fujikuroi* genes.

4.2. Cross-pathway control is independent of Mbf1 in F. fujikuroi

The *mbf1* gene which had been already identified as up-regulated in the GS mutant (Teichert et al., 2004), was now shown to be a direct Cpc1 target gene. It belongs to a family of multi-protein bridging factors highly conserved from yeast to higher eukaryotes. In yeast, they mediate the binding of stress-responsive transcription factors to the TATA-box binding proteins. In *Arabidopsis thaliana*, three different *mbf1* homologues exist which are all able to complement a *S. cerevisiae mbf1* mutant, highlighting the strong functional conservation of these proteins across the kingdoms (Tsuda et al., 2004). Homologues had been shown to be co-regulated with *cpc1* in *Trichoderma reesei* under secretion stress (Arvas et al., 2006) and in the *F. fujikuroi areA* deletion mutant under nitrogen starvation (Schönig et al., 2008). In contrast, *mbf1* was not identified as a *S. cerevisiae* GCN4 target gene (Kellis et al., 2003; Natarajan et al., 2001).

Plate assays and northern blot expression analysis of Cpc1 target genes clearly showed that the deletion of *mbf1* did not result in a Cpc1-like phenotype and had no effect on Cpc1 target gene expression (Fig. 3, Table 3, Fig. 4). Additionally, no interaction between Cpc1 and Mbf1 was detected by using two different yeast two-hybrid systems (Fig. S2), although it might be possible that the fusion of additional domains required for the yeast two-hybrid screenings alter the tertiary structure of the proteins in a way that prevents interaction with other proteins. *In vitro* assays, such as pull-down analysis or co-immuno precipitation, might be necessary to give a definitive answer as to whether Mbf1 and Cpc1 interact. In contrast to *A. thaliana*, no other potential MBF1 homologues can be identified in the genomes of the closely related *F. verticillioides* and *Fusarium oxysporum* (www.broad.mit.edu/annotation/genome/fusarium_verticillioides/MultiHome.html), or in other sequenced fungal genomes making it unlikely that a redundant protein acts as Cpc1 co-activator in the Δ*mbf1* mutant. This evidence strongly suggests that Mbf1 is not required for activation of CPC1 target genes in *F. fujikuroi*.

4.3. Is there a cross-talk of nitrogen metabolite repression and cross-pathway control?

Under conditions of nitrogen starvation, *cpc1* expression is very weak, but increases significantly when nitrogen sources are added. Amino acid starvation, provoked either by addition of inhibitors of amino acid biosynthesis or mutation of amino acid biosynthetic genes, enhanced *cpc1* expression even more markedly. Cpc1 target gene expression mirrors the expression of *cpc1* itself, indicating that Cpc1 activity is rather modulated at the transcriptional than at the translational level in *F. fujikuroi*. Amino acid biosynthetic genes are in most cases (except for *glnA*) expressed in the same manner as *cpc1* itself; they are neither repressed by the end product of their biosynthetic pathway, nor specifically expressed in response to starvation of their specific end product (Fig. 6). The induction of *cpc1* expression by amino acid analogues overrides the repression of *cpc1* transcription under nitrogen starvation (Figs. 4–6). The extremely elevated transcript level of *cpc1* and its target genes upon addition of a single amino acid might be due to amino acid imbalances in the cell, as shown for *S. cerevisiae* and *N. crassa* (Barthelmess, 1986; Niederberger et al., 1981).

In a recent macroarray approach (Schönig et al., 2008) and in this work (Fig. 4), we have shown that *cpc1* is significantly up-regulated in a Δ*areA* mutant under nitrogen starvation conditions. AreA is known as a positively acting transcription factor in fungi which is responsible for the up-regulation of nitrogen-scavenging genes when preferred nitrogen sources, such as ammonium and glutamine, are not available. Recently, we showed that AreA might also act as a repressor (Schönig et al., 2008) what might be the case for *cpc1* under nitrogen starvation conditions. However, a putative interconnection between cross-pathway control (CPC) and nitrogen catabolite repression (NCR) remains ambiguous in fungi. In *S. cerevisiae*, there are indications for a role of GCN4 in NCR by inhibiting GLN3, the homologue of AreA, and for inhibition of GCN4 translation under nitrogen starvation (Grundmann et al., 2001; Sosa et al., 2003). In filamentous fungi, nothing is known so far about the coordination of nitrogen metabolite repression and cross-pathway control except that glutamine limitation is a weaker elicitor of the CPC in *N. crassa* (Kolanus et al., 1990). A direct repression of *cpc1* transcription by binding of AreA to the 5′ UTR of *cpc1* seems unlikely, since the promoter of *cpc1* contains only one single GATA sequence element, the AreA-binding site. The AreA homologues in several fungi were shown to preferentially bind to double GATA elements (Marzluf, 1997) as has been confirmed also for the *F. fujikuroi* AreA, which activates GA gene expression by binding to double GATA elements in the 5′ leader sequence (Mihlan et al., 2003). Thus, the mechanism of regulation of

Table 3
Growth of Δ*cpc1* is retarded by amino acid imbalances.

Strain	Colony ∅ (mm)					
	Gln		$(NH_4)_2SO_4$		HSer	Ile
	$-H_2O_2$	$+H_2O_2$	−MSX	+MSX		
WT	20.7 ± 0.4	12.7 ± 0.4	12.0 ± 0.5	9.7 ± 0.4	10.2 ± 0.4	15.0 ± 0
Δ*cpc1*	21.2 ± 0.4	13.0 ± 0.5	12.0 ± 0.5	0 ± 0	5.7 ± 0.4	8.0 ± 0
$cpc1^{comp}$	20.0 ± 0	13.5 ± 0.5	12.0 ± 0.5	10.0 ± 0.5	10.2 ± 0.4	15.0 ± 0
Δ*mbf1*	21.0 ± 0.5	13.5 ± 0.5	12.5 ± 0.5	10.0 ± 0	10.0 ± 0	13.7 ± 0.4

Strains were grown for 3 d at 28 °C. (mm): diameter of colonies in millimeter. Four different sets of plates were analysed. For media details see Fig. 3.

cpc1 expression by AreA remains unclear, although binding to a single GATA element cannot be excluded.

Down-regulation of *cpc1* expression under nitrogen starvation corresponds to regulation of cellular localization of Cpc1: the GFP-Cpc1 fusion protein fails to localize in the nucleus under nitrogen starvation, but accumulates in the cytoplasm. The addition of a single amino acid, e.g. glutamate, as sole nitrogen source, or of the GS inhibitor MSX, results in translocation of Cpc1 to the nucleus (Fig. 7). These findings agree with data obtained from *Aspergillus fumigatus*, where it had been found that a CpcA-GFP fusion protein expressed from the endogenous *cpcA* promoter was generally not expressed in a murine model of pulmonary aspergillosis, but is expressed and localized in the nucleus in the presence of 3-AT (Sasse et al., 2008). The cytoplasmic localization of Cpc1 under nitrogen starvation in *F. fujikuroi* demonstrates that Cpc1 activity is also regulated at the post-translational level beside regulation of *cpc1* expression, probably by inhibiting its accumulation in the nucleus, or by regulated export from the nucleus. This inhibition induced by nitrogen starvation is lifted at the onset of starvation for a specific amino acid, probably to ensure availability of all proteinogenic amino acids. Thus, we suggest that the cross-pathway control transcription factor Cpc1 is regulated at least on two levels: first of all on the transcriptional level, as *cpc1* transcription is repressed under nitrogen starvation in an AreA-dependent manner and induced by oversupply of, or starvation for, a single amino acid; and secondly on protein level as Cpc1 activity is also regulated by controlling the cellular localization of Cpc1 in a yet unknown mechanism.

4.4. Secondary metabolism is independent of Cpc1 in F. fujikuroi

Since the production of secondary metabolites is completely abolished in the *glnA* mutant (Teichert et al., 2004), the question arises whether Cpc1 might be responsible for the down-regulation of secondary metabolism genes under conditions of amino acid starvation. For *A. nidulans* it has been postulated that CpcA down-regulates penicillin production to favour lysine biosynthesis under conditions of amino acid limitation (Busch et al., 2003). If this hypothesis was correct for *F. fujikuroi*, the transcription of the GA gene *cps/ks* would have to be de-repressed by double *cpc1*/*glnA* deletion, or in the Δ*cpc1* mutant supplemented with the specific GS inhibitor MSX. However, *cps/ks* is not de-repressed in the *cpc1* deletion mutant treated with MSX, excluding the possibility that Cpc1 may be involved in the regulation of secondary metabolism by GS (Fig. 1). A possible explanation for this contrasting result compared to the up-regulation of penicillin biosynthesis in *A. nidulans* in a CpcA mutant is the fact that the biosynthesis of GAs does not interfere with amino acid biosynthesis as it is the case for penicillin. Therefore, other regulatory mechanisms than the cross-pathway control network have to exist that respond to the deletion of the *glnA* gene in *F. fujikuroi* and repress GA and bikaverin biosynthesis genes.

One of the most interesting results in this work is the finding that the expression of the *glnA* gene does not differ between the wild-type and the Δ*cpc1* mutant as it is the case for the other amino acid biosynthetic genes. Therefore, *glnA* is not a Cpc1 target gene in *F. fujikuroi* in contrast to *N. crassa* (Tian et al., 2007) (Fig. 4). Its expression is clearly up-regulated by MSX addition in both strains suggesting that there must be another regulator inducing *glnA* expression under glutamine starvation conditions. This regulator might be AreA, since the expression of *glnA* decreases significantly in the Δ*areA* mutant and induction by MSX is not longer observed (Fig. 4). AreA may up-regulate *glnA* transcription to compensate for decreasing glutamine levels inside the cell. In contrast, expression of the secondary metabolism gene *cps/ks* is not up-regulated by MSX addition, but completely lost, which argues strongly for a central role of the GS in nitrogen metabolite repression and regulation of secondary metabolism.

Moreover, analysis of amino acid auxotrophic mutants showed that the Δ*glnA* mutant is the only auxotrophic mutant that lost the ability to produce secondary metabolites. In the other mutants, transcription of the GA gene *cps/ks* is unchanged or even elevated as in the methionine auxotrophic mutant. This is an additional indication that GS plays a central role in nitrogen regulation.

In conclusion, the present study identifies Cpc1 as the major transcription factor essential for the response to the amino acid status of the cell. The *F. fujikuroi* Cpc1 transcription factor is not responsible for the down-regulation of secondary metabolism in the Δ*glnA* mutant or under MSX addition, and it does not require the Mbf1 protein for activation of cross-pathway control target genes. Cpc1 expression is negatively regulated under conditions of nitrogen starvation by transcriptional as well as post-translational mechanisms: *cpc1* transcription is repressed under nitrogen starvation in an AreA-dependent manner, and localization of Cpc1 in the nucleus is abolished during nitrogen limitation. This nitrogen-dependent inhibition of nuclear translocation is overruled by amino acid limitation, since amino acid starvation induced by addition of amino acid analogues leads to nuclear accumulation of Cpc1. The activation of Cpc1 is also mirrored by an increase of *cpc1* mRNA levels, not only by amino acid depletion, but also by oversupply of single amino acids, indicating that any kind of amino acid imbalance triggers the onset of cross-pathway control.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2009.08.003.

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