

The COT1 homologue CPCOT1 regulates polar growth and branching and is essential for pathogenicity in *Claviceps purpurea*

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Abstract

Claviceps purpurea, the ergot fungus, is a common grass pathogen attacking exclusively young ovaries. Its pathogenic development involves an early phase of directed growth (with strictly suppressed branching) towards the floral vascular tissue. Since Ser/Thr protein kinases of the NDR family have been shown to be involved in polar growth and branching in fungi, we have analyzed a *C. purpurea* homologue of the *Neurospora crassa cot-1* gene, *cpcot1*. It encodes a functional homologue of COT1 since it can fully complement the *N. crassa cot-1* mutant phenotype. $\Delta cpcot1$ mutants are significantly impaired in vegetative growth properties: they are characterized by hyperbranching, reduced growth rate, and decreased conidiation. Infection studies on rye plants and isolated ovaries show that the $\Delta cpcot1$ mutants are apathogenic; microscopical analyses indicate a very early block, probably in penetration. Thus CPCOT1 is not only involved in polarity and branching and hence oriented growth in the host tissue as expected, but it is essential for the initiation of infection.

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1. Introduction

The typical hyphal growth of filamentous fungi requires establishment and maintenance of hyphal polarity and a defined branching pattern. These processes have been studied intensively in model fungi like *Neurospora crassa* and *Aspergillus nidulans* (for recent review see Harris and Momany, 2004); major players in these processes are Ca²⁺-dependent signaling components (Zelter et al., 2004) and general factors involved in organization of the cytoskeleton. Among different *N. crassa* mutants that have been described which are impaired in polar growth and/or branching, *cot-1* has been most extensively studied (Gorovits and Yarden,

2003; Gorovits et al., 1999, 2000; Yarden et al., 1992). COT1 is a Ser/Thr protein kinase related to the NDR family, which includes several important kinases involved in cell-cycle regulation and morphogenesis (Tamaskovic et al., 2003). COT1 shows significant homology to the WARTS kinase of *Drosophila melanogaster*, ORB6 of *Schizosaccharomyces pombe*, Cbk1 of *Saccharomyces cerevisiae* and the human NDR kinase. Two homologues so far have been identified in pathogenic fungi: UKC1 from *Ustilago maydis* (involved in morphogenesis, pathogenesis, and pigmentation; Durrenberger and Kronstad, 1999) and TB3 from *Colletotrichum trifolii* (involved in hyphal elongation and branching; Buhr et al., 1996). We describe here the cloning and functional analysis of the *cot-1* homologue *cpcot1* from the ascomycete *Claviceps purpurea*, a common pathogen of grasses and cereals worldwide.

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The early pathogenic development of *C. purpurea* (for reviews see Oeser et al., 2002a; Tudzynski and Tenberge, 2003; Tudzynski and Scheffer, 2004) involves at least two phases, in which polarity and reduced branching could be essential: in the first hours after attachment of conidia on the stigmatic hairs of the grass flower (*C. purpurea* is highly organ specific!), the germination tubes grow only a short distance (more or less unbranched) on the surface and then penetrate the host's epidermis, a process which requires reorientation and establishment of polarity; specific infection structures are not formed. After penetration, the fungus follows the path of the pollentube and grows in thick hyphal bundles (almost unbranched) through the stigma and the ovarian tissue towards the basis of the floret, tags the vascular tissue, and only then starts to colonize the ovarian tissue with normal branching pattern.

We have previously shown that two MAP kinases, the *fus3* homologue CPMK1 and the *slt2*-homologue CPMK2, are involved in the penetration phase; mutants lacking either of these kinases are completely apathogenic and unable to penetrate (Mey et al., 2002a,b). On the other hand, we demonstrated that pectin degradation is essential for the early colonization phase: double mutants lacking two endopolygalacturonase genes (*cpgg1*, *cpgg2*) are able to penetrate the epidermal tissue, but cannot colonize the host tissue, i.e., they are also fully apathogenic (Oeser et al., 2002b). Here we show that the *C. purpurea* COT1 homologue—CPCOT1 significantly affects growth morphology and branching in this pathogen, and that it is essential for very early stages of infection, probably even during the penetration phase. Our results determine that the establishment of hyphal polarity and the control of branching are essential prerequisites for the pathogenic development of this flower pathogen.

2. Materials and methods

2.1. Strain media and growth conditions

The wild-type *C. purpurea* strain used in these experiments was 20.1, a putative haploid derivative of standard field isolate T5 (Fr.:Fr.) Tul., isolated from rye (*Secale cereale* L.; Hohenheim, Germany), and obtained by benomyl treatment (Hüsken et al., 1999). For conidia harvesting and DNA isolation, mycelia were cultivated on Mantle agar (16 g/L agar) with 100 g/L sucrose (Mantle and Nisbet, 1976) at 28 °C for 12–14 days. *Neurospora crassa* wild-type strain 74-OR23-1A (FGSC 987) and *cot-1* (FGSC 4065) were used throughout this study. Procedures used for fungal growth and other manipulations were described by Davis (2000). Strains were grown in either liquid or solid (supplemented with 1.5% agar) Vogel's medium with 1.5% (w/v) sucrose. When appropriate, the medium was supplemented with 100 µg/ml

hygromycin B (Calbiochem or Boehringer–Mannheim). *Escherichia coli* strain TOP10F' (Invitrogen) was used for all the subcloning experiments. *E. coli* strain LE392 (Stratagene) was employed for propagation of *C. purpurea* genomic lambda clones.

2.2. Nucleic acid extraction and analysis

Standard recombinant DNA methods were performed according to Sambrook et al. (1989) and Ausubel et al. (1987). Genomic DNA from *C. purpurea* was prepared from lyophilized mycelium according to Cenis (1992). For Southern blot analysis, 5–10 µg of restriction-digested chromosomal DNA or PCR products were electrophoresed in 0.8–1.6% agarose gels with salt-free buffer (Sambrook et al., 1989), blotted onto positively charged nylon filters (Hybond-N+; Amersham, Braunschweig, Germany), and hybridized to radioactivity-labeled DNA probes in Denhardt's hybridization solution (Sambrook et al., 1989). Filters were washed for 10 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) and for 10 min in 1× SSC, 0.1% SDS. The hybridization and washing temperatures used were 57 and 65 °C for low and high stringency conditions, respectively. DNA sequencing was carried out as described by Moore et al. (2002). Protein and DNA sequence alignment, editing, and organization were done with DNA Star (Madison, WI, USA). Sequence analysis was done using BLAST at National Center for Biotechnology Information, Bethesda, MD, USA (Altschul et al., 1990). PCR was done as described by Sambrook et al. (1989), using the Red *Taq* Polymerase (Sigma, Milwaukee, WI, USA). All primers were synthesized by MWG-Biotech (Mnchen, Germany). The amplification products were cloned with the PCR 2.1 TOPO-Cloning Kit from Invitrogen. *N. crassa* genomic DNA and RNA were isolated and manipulated as previously described (Yatzkan et al., 1998). RNA samples were treated with RQ1 RNase free DNase (Promega, Madison, WI, USA) and then purified with RNeasy kit (QIAGEN, Hilden, Germany) according to manufacturer's protocol. RT reaction was performed using SuperScript II RNase H- reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

2.3. Cloning of *cpcot1* and generation of a replacement vector

A 716 bp internal fragment of *cpcot1* was amplified by PCR using the primers (deducted from the *N. crassa cot-1*-sequence) COT1A (5'-ATCGGCAAGGGCGCTTTCGGC-3') and COT1B (5'-CCGGAGCAATATAATCTGGTGTACC-3') and genomic DNA from *C. purpurea* strain 20.1. The amplification product, cloned into the PCR 2.1 TOPO vector (Invitrogen), was excised with an *EcoRI* restriction and used as a probe to screen a genomic library of strain T5 (Smit and Tudzynski, 1992) by plaque

filter hybridization (Sambrook et al., 1989). From the 34,000 lambda clones screened, 13 hybridized to the PCR probe, and four of them were further purified (Sambrook et al., 1989). They all contain an overlapping genomic region, as revealed by restriction and southern blot analyses. From the phage No. 3 a 10kb *SalI* fragment and a 2.5kb *EcoRI* fragment were cloned into pUC19 and a 4kb *EcoRV* fragment was cloned into pBSK⁻, giving the pCOTS1, the pCOTEI1, and the pCOTEV1 plasmids. A 1.5kb *XbaI*–*SalI* fragment of pCOTS1 and a 1.4kb *HindIII* fragment of pCOTEV1 were subcloned into pUC19 yielding the pCOTXS1 and pCOTH1 plasmids, respectively. pCOTXS1, pCOTEI1, and pCOTH1, overlapping and carrying the complete *cpcot1* gene, were completely sequenced.

For the construction of the *cpcot1* replacement vector, the genomic regions upstream and downstream of *cpcot1* (–332/+535 bp relative to the start codon and +92/+966 bp relative to the stop codon), were amplified by primers with integrated restriction sites: for the amplification of the upstream flank *HindIII* (within primer DCOT-1) and *BglII* (DCOT-2) and for the downstream flank *XbaI* (DCOT-3) and *SacII* (DCOT-4). These PCR products were cloned into the PCR 2.1 TOPO vector, excised with *HindIII*–*BglII* and *XbaI*–*SacII*, respectively, and subcloned upstream and downstream the phleomycin resistance cassette (*ble*) into the corresponding restriction sites of the pAN8-1UM (Müller et al., 1997) vector, producing the pΔ*cpcot1* plasmid. The linear replacement construct was excised using *HindIII*–*SacII* and subsequently used to transform the *C. purpurea* wild-type strain 20.1 (see Fig. 3).

To obtain a full-length clone of *cpcot1* for complementation of the *N. crassa cot-1* mutant a 0.9kb *XbaI*–*EcoRI* fragment of pCOTXS1 and the 2.6kb fragment of pCOTEI1 were fused into pBluescript. The resulting construct was designated pCPCOT1. Thus, pCPCOT1 carries the coding sequence of *cpcot1* and 0.6kb of its 5' and 0.5 kb of its 3' flanking regions. The region spanning the ligation site used for fusing the two *cpcot1* fragments was sequenced to verify no changes were introduced during the construction process. The complementation vector for *C. purpurea cpcot1* mutants was obtained by cloning a 2.6kb *XbaI* resistance cassette for hygromycin from the pLOF1A plasmid (kindly provided by J. van Kan, University of Wageningen, The Netherlands) into the corresponding restriction site of the pCPCOT1 vector. This vector was termed pCOTCOMP and was used to transform the *C. purpurea cpcot1* mutant strain Δ*cpcot1*-1.

3. Fungal transformation

Protoplasts of *C. purpurea* generated with lysing enzymes from *Trichoderma harzianum* and Driselase

(InterSpex) were transformed with 10 μg of the pΔ*cpcot1* fragment (see Fig. 3) as described by Jungehülsing et al. (1994). For phleomycin selection, phleomycin was directly applied to the protoplasts to a final concentration of 33 μg/ml modified BII medium (pH 8, 20% saccharose, no FeSO₄). Resistant colonies were transferred to fresh selective medium (BII pH 8 + 100 μg/ml phleomycin) and subjected to at least one round of single spore isolation to obtain homokaryotic transformed strains.

The phleomycin-resistant transformants carrying a homologous integration of the replacement construct were identified by PCR, using primers DCOT-hIL1 (5'-AAAAGCGGTGATTGGGATTGAA-3') and DCOT-hIL2 (5'-GTTACGCCGTCTGACTTTTGTGGT-3') for the left flank and DCOT-hIR1 (5'-TCCGGCGAA GAGAAGAATAGC 3') and DCOT-hIR2 (5'-GCGC CATAAACTCATAACCCATAA-3') for the right flank (shown in Fig. 3) and 10–100 ng of genomic DNA. The predicted 1980 and 1473 bp fragments were amplified with the strains E51 and E83. The lack of the wild-type gene copy in the Δ*cpcot1* mutants was checked using the primers DCOT-WT1 (5'-ATGGAAGA GTCGCCGAGATGAGC-3') and DCOT-WT2 (5'-C GAAACCCGCACACCGAGAACC-3'), which gave rise to a 1243 bp fragment with the wild-type strain.

For complementation of the *C. purpurea cpcot1* mutant, Δ*cpcot1*-1 was transformed with the circular pCPCOT1 construct. For hygromycin selection protoplasts were incubated at 28 °C for 24 h, after which they were overlaid with 10 ml BII medium pH 8 containing 1.5 mg/ml hygromycin to reach a final hygromycin concentration of 0.5 mg/ml in the petri dishes. Hygromycin resistant transformants were purified by single spore isolation and checked for the reintegration of *cpcot1* by PCR with the primers DCOT-hIL1 and DCOT2 and primers COTKOMP1 (5'-CCACACGGCACAATACT CCTC-3') and COTKOMP2 (5'-TTCTCAAACGCT GCCAATACAAG-3'; for location, see Fig. 3) and by Southern blot analysis.

For complementation of the *N. crassa cot-1* mutant, the pCPCOT1 construct was linearized with *XbaI* and co-transformed by electroporation to *cot-1* conidia, along with a construct harboring a cassette conferring Hygromycin resistance (pMP6, kindly provided by M. Plamann). Electroporation was performed according to Margolin et al. (1997). The transformants were screened by their resistance to Hygromycin and by phenotypic complementation of *cot-1* (ability to grow at the restrictive temperature –34 °C).

Hygromycin resistant transformants were purified by single spore isolation and examined for the integration and expression of *cpcot1* by PCR and RT-PCR, respectively, with the primers *cpcot1*-2222L (5'-GAAGGCT CTT GGCCTATTCC-3') and *cpcot1*-2921R (5'-GCT TTCATAT GCCCACACAA-3'). The PCR and RT-

PCR products were sequenced to verify the synthesis of the required amplicon. The *cot-1* PCR and RT-PCR control was obtained using the primers cot1-2886L (5-ATCAAGAGCC ACGCCTTCT-3) and cot1-3835R (5-CGTATCGCGG GCATAGTATT-3). The β -tubulin PCR and RT-PCR control was obtained using the primers btub1304L (5-CCGTCTCCAC TTCTTCATG G-3) and btub1862R (5-AGCATCCTGG TACTGCTG GT-3).

3.1. Light microscopy observations of hyphal tips

Claviceps purpurea wild-type strain 20-1, the ectopic transformant E87, the Δ *cpcot1* mutants (Δ *cpcot1*-1 and Δ *cpcot1*-2) and the complemented strain CC5 were grown on glass slides covered with different media at 28 °C at high humidity. The edge of the growing colonies was directly observed 4 dpi using light microscopy and photographed. For *N. crassa* light microscopy, samples were viewed with a Zeiss Axioscope microscope. Documentation was performed with a DVC C300 digital camera.

3.2. Pathogenicity tests

Rye plants were cultivated in growth chambers as described by Smit and Tudzynski (1992). Florets of blooming ears (30–40/ear) were inoculated with 5 μ l of a suspension containing 2×10^6 /ml conidia collected from Mantle agar, as described by Tenberge et al. (1996). To avoid cross contamination, the ears were covered with paper bags equipped with cellophane windows directly after inoculation. To introduce the fungus inside the plant (“wounding test”), rye florets were bisected and inoculated with the conidial suspension. Pictures were taken 4 weeks post-inoculation.

3.3. Aniline blue staining and fluorescence microscopy observations

For this analysis an in vitro infection system was used (modified from Heslop-Harrison and Heslop-Harrison, 1981, J. Scheffer, unpubl.): rye pistils were isolated from blooming rye ears and put onto Hoagland solution (Hewitt, 1966; Hoagland and Arnon, 1938) modified for barley shoot culture [for 1 L: Mes buffer 5 ml (2-morpholino ethansulfonic acid 19.5 g/L, NaOH 2.0 g/L, pH 6.5), Ca(NO₃)₂·4H₂O 94 mg, KNO₃ 66 mg, MgSO₄·7H₂O 52 mg, KH₂PO₄ 38 mg, H₃BO₃ 2.86 mg, ZnSO₄·7H₂O 0.22 mg, CuSO₄·5H₂O 0.1 mg, Na₂MoO₄·2H₂O 0.05 mg, and FeEDTA 12 mg] and solidified with 16 g/L agar. Stigmas were infected with suspensions containing 2×10^6 conidia/ml with an inoculation loop and were incubated at RT for 5 days.

The KOH-aniline blue staining of the fungus was realized as described by Hood and Shew (1996), except

that the pistils were incubated in 1 M KOH overnight at room temperature. The infected ovaries were observed using epifluorescence microscopy (Microscope Leica DMRBE with PixelFly Digital camera (PCO Computer Optics GmbH), filter block A (BP 340-380, RKP 400, LT 425), UV light 340–380 nm).

4. Results

4.1. *Cpcot1* encodes a COT1-homologous protein kinase

For the cloning of a *cot-1* homologue, a PCR-fragment showing significant homology to *cot-1* was used as probe to screen a genomic EMBL3 library of *C. purpurea* (for details, see Section 2). Based on Southern blot analysis of lambda phages potentially harboring the *cot-1* homologue, followed by subcloning and sequence analysis, an open reading frame of 1968 bp, interrupted by three introns of 127, 94, and 100 bp (see Fig. 3A) was identified, encoding a polypeptide of about 74 kDa. Southern blot analyses under low stringency conditions, using digested genomic DNA from the wild-type strain 20.1 and, as a probe, the PCR product used for the screening of the genomic library revealed one 9 kb *Hind*III fragment, two *Eco*RV fragments of 8 and 2.8 kb, and two *Pst*I fragments of 8 and 0.7 kb (data not shown). These results are consistent with the presence of a single copy of the *cpcot1* gene in the genome of *C. purpurea*. The derived amino acid sequence showed high homology to COT1 and the gene was termed *cpcot1*. A phylogenetic tree analysis (Fig. 1) groups CPCOT1 together with other fungal Ser/Thr kinases, the highest homology being a hypothetical protein FG01188.1 from *Giberella zeae* (77.7% identity), TB3 from *C. trifolii* (76.7%), and COT1 from *N. crassa* (74.2%).

A direct alignment between CPCOT1 and its homologues COT1 from *N. crassa*, TB3 from *C. trifolii* and FG01188.1 from *G. zeae* demonstrate the high degree of conservation between these protein kinases (data not shown). All of the conserved serine/threonine kinase motifs (Hanks et al., 1988) identified in COT1 are present in CPCOT1. Interestingly, CPCOT1—like TB3—has a glutamine stretch in the amino terminal regulatory domain, which is lacking in COT1 or ORB6 from *S. pombe* (but also present in WARTS of *D. melanogaster*). This glutamine-rich region is considered to be involved in nuclear localization and in transcriptional activation (Chen and Dickman, 2002). Moreover, as in TB3, a potential nuclear localization signal at residues 223–226 (K(R/K)X(R/K), in which X is K, R, P, V, or A; Chelsky et al., 1989) was identified in CPCOT1. Using RT-PCR, expression of *cpcot1* could be detected throughout pathogenic development (data not shown).

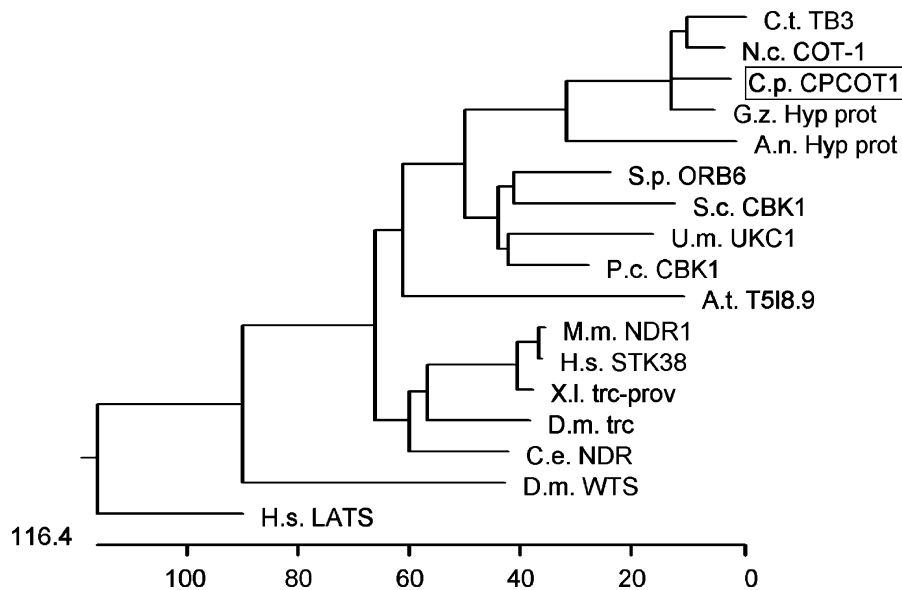


Fig. 1. Phylogenetic tree of the NDR kinase family. A.n. Hyp prot: *Aspergillus niger* (EAA62689); A.t. T518.9: *Arabidopsis thaliana* (G86431); C.e. NDR: *Caenorhabditis elegans* (CAA84441); C.p. CPCOT1: *Claviceps purpurea* (AJ784154); C.t. TB3: *Colletotrichum trifolii* (S70706); D.m. trc: *Drosophila melanogaster* (NP_524170); D.m. WTS: *D. melanogaster* (Q8T0S6); G.z. Hyp prot: *Gibberella zeae* PH-1 (XP_381364); H.s. LATS: *Homo sapiens* (NP_004681); H.s. STK38: *H. sapiens* (NP_009202); M.m. NDR1: *Mus musculus* (AAP44997); N.c. COT-1: *Neurospora crassa* (P38679); S.c. CBK1: *Saccharomyces cerevisiae* (NP_014238); S.p. ORB6: *Schizosaccharomyces pombe* (NP_593165); U.m. UKC1: *Ustilago maydis* (AAC09291); X.l. trc-prov: *Xenopus laevis* (AAH56129).

4.2. CPCOT1 is a functional homologue of COT1

To test if the gene product of *cpcot1* can functionally replace COT1 in *N. crassa*, a *cot-1* mutant was transformed with a full-length genomic copy of *cpcot1*, including 586 bp of upstream non-coding sequences. As shown in Fig. 2A, for two of the several transformants obtained, the *C. purpurea* gene can fully restore wild-type growth and normal branching in the *cot-1* mutant, proving that CPCOT1 is a functional homologue of COT1. None of the control strains (transformed only with the Hygromycin resistance cassette) exhibited improved hyphal growth at the restrictive temperature. The presence of the *cpcot1* coding sequence in the transformants (and not in wild-type of *N. crassa* and *cot-1*) was determined by PCR analysis (Fig. 2B), and the expression of *cpcot1* in the transformants was verified by RT-PCR (Fig. 2C).

4.3. Generation of $\Delta cpcot1$ mutants

To analyze the function of CPCOT1 in *C. purpurea*, $\Delta cpcot1$ mutants were generated using a gene replacement approach. As outlined in Fig. 3, 5'- and 3'-fragments of the gene were cloned upstream and downstream of the phleomycin resistance cassette of pAN8-1UM (details see Section 2). The replacement fragment was excised from the resulting vector p $\Delta cpcot1$ and used for transformation of *C. purpurea* strain 20.1. Phleomycin-resistant transformants were screened by diagnostic PCR (using primers DCOT1-hIL1/DCOT1hIL2 and DCOT1hIR1/DCOT1hIR2, see Fig. 3) for the presence of a replacement

fragment at the *cpcot1* locus (originated from double crossover). Two of the total 870 transformants showed this fragment, indicating a homologous integration rate of 0.2%. Since these transformants still contained the wild-type fragment (obtained with primers DCOT1-WT1/DCOT1-WT2, see Fig. 3A), they were genetically purified by two rounds of single-spore-isolation. Two independent mutants showing only the diagnosing PCR fragment (data not shown) were obtained and named $\Delta cpcot1-1$ and $\Delta cpcot1-2$. The replacement event was confirmed by Southern blot analysis (Fig. 4), demonstrating that the mutants clearly lack the 1.4 kb wild-type fragment hybridizing to the *cpcot1* probe; as expected the probe had hybridized to a larger DNA fragment (5.5 kb).

To verify that the effects observed in the $\Delta cpcot1$ mutant (see below) were indeed caused by inactivation of *cpcot1*, $\Delta cpcot1-1$ was used as recipient for transformation with the full-length genomic copy of *cpcot1* (for details see Section 2); the presence of the complete *cpcot1* sequence was confirmed by PCR (data not shown) and Southern blot analysis (Fig. 4): the complemented strain CC5 shows an additional fragment of 0.5 kb as expected (see Fig. 3B).

4.4. Characterization of $\Delta cpcot1$ mutants

On solid medium $\Delta cpcot1$ mutants show a distinct phenotype: their growth is significantly retarded, and the colony morphology is “popcorn”-like (see Fig. 5). The mycelium tends to avoid growing on and in the medium; mostly aerial hyphae are formed. On agar-coated glass-

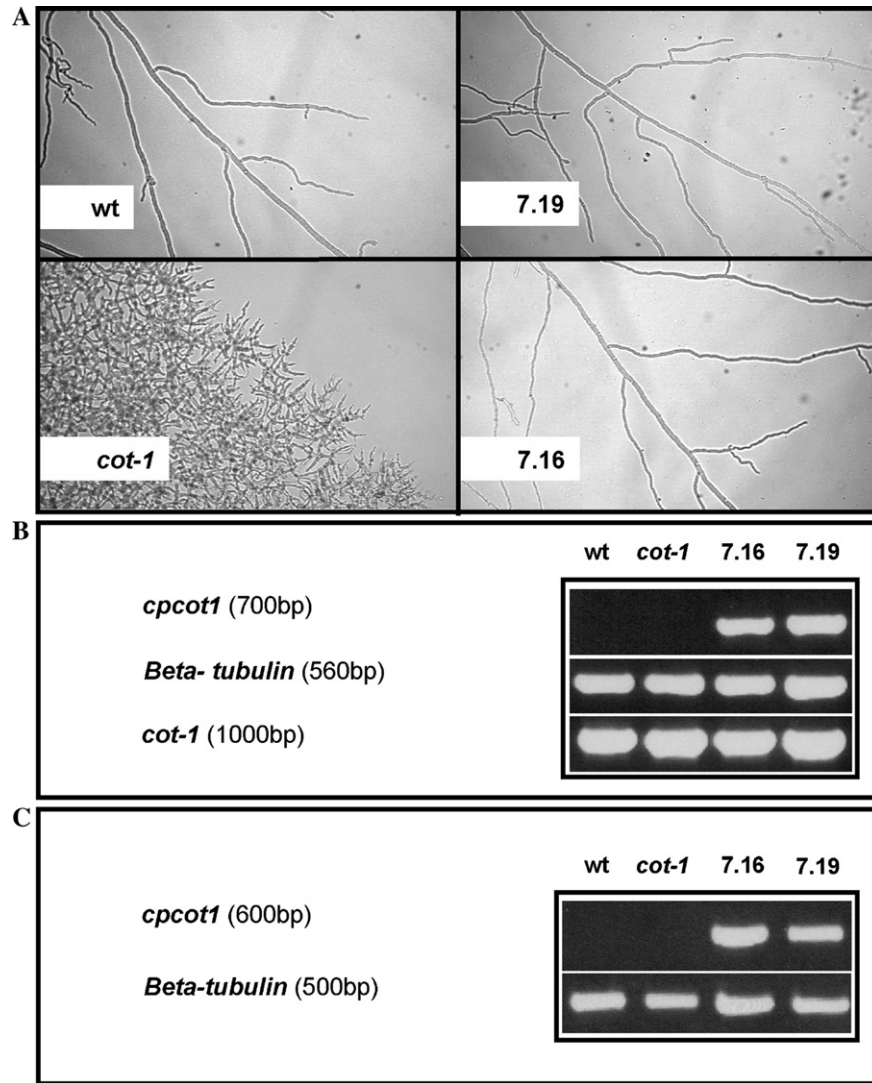


Fig. 2. (A) Morphology of wild-type, *cot-1* and two *N. crassa cpcot1* transformants cultured at 37 °C. (B) Presence of *cpcot1*, β -tubulin, and *cot-1* DNA in wild-type, *cot-1* and two *N. crassa cpcot1* transformants, as determined by PCR (amplicon size is noted in parentheses). (C) Presence of *cpcot1* and β -tubulin transcripts in wild-type, *cot-1* and two *N. crassa cpcot1* transformants, as determined by RT-PCR (amplicon size is noted in parentheses).

slides the hyphae show an aberrant morphology (Fig. 6), comparable to the *cot-1* phenotype in *N. crassa* (Fig. 2A): the hyphae are short, coiled, and hyperbranched. Hyphal tips are club-shaped. This indicates that CPCOT1—like COT1—is involved in polar growth and branching of hyphae. In addition, conidia vary in size and form, but they show a germination rate, which is comparable to the wild-type (data not shown). Complementation with the intact *cpcot1* gene fully restores wild-type morphology including the cell shape (Fig. 6) and the size of the conidia, confirming that the observed mutant phenotype is due to inactivation of *cpcot1*. As the *cot-1* phenotype in *N. crassa* can be suppressed by a change in external osmolarity (Gorovits and Yarden, 2003) we tested our mutants on media containing 0.5–1.5 M NaCl and 0.75–1.5 M sorbitol. Radial growth of the wild-type was inhibited at concentrations above 1 M NaCl and

1 M sorbitol, respectively. At concentrations below 1 M, growth was not significantly affected. In contrast to the *N. crassa cot-1* mutants, no suppression of the $\Delta cpcot1$ mutant phenotype could be observed with any concentration tested (data not shown).

To elucidate whether the club-shaped form of the tips and the tendency to form aerial hyphae is due to any instability of the cell wall, we embedded the wild-type and the $\Delta cpcot1$ mutants in media with different concentrations of agarose. However, the mutants had no defect in invasive growth in media containing up to 8% agarose, which represents a material resistance comparable to compression of about 0.1 MPa (Brush and Money, 1999). Interestingly, during invasive growth of the mutants a partial restoration of the wild-type phenotype was observed; hyphal cells were elongated and not club-shaped (Fig. 7).

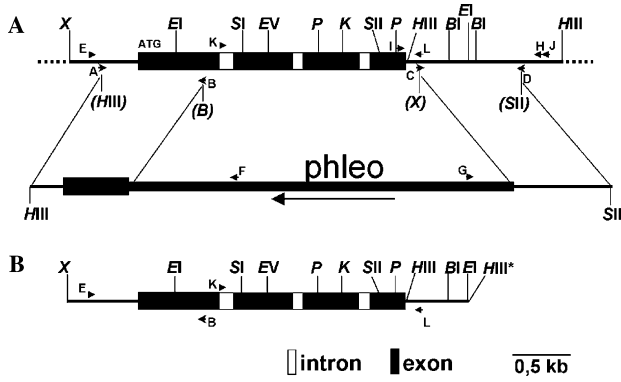


Fig. 3. Gene replacement approach (A) and complementation construct (B) for *cpcot1*. (A) The replacement vector pΔ*cpcot1* was constructed by cloning 3' and 5' parts of *cpcot1* on each side of a phleomycin resistance cassette (*ble*) in the pAN8-1UM plasmid (see Section 2 for details). The resistance cassette, excised using a *Hind*III–*Sac*II restriction, was used to transform the *C. purpurea* wild-type strain 20.1. Δ*cpcot1* mutants were generated following the disruption of the wild-type gene (WT) by homologous recombination through a double crossover event. The *cpcot1* coding sequence and introns are represented with black and white boxes, respectively. The ATG indicates the start of transcription of *cpcot1* and the black arrow, the orientation of the phleomycin resistance cassette within the replacement construct. Primers DCOT-1/DCOT-2 (indicated as A, B) and DCOT-3/DCOT-4 (C and D) used for the amplification of the upstream and downstream flanks, respectively, are indicated with black arrows. The positions of primers DCOT-hIL1/DCOT-hIL2 (E and F) and DCOT-hIR1/DCOT-hIR2 (G and H), used for the identification of homologues integrations and primers DCOT-WT1/DCOT-WT2 (I and J), used for detection of *cpcot1* wild-type copy, are indicated with black triangles. (B) Full-length clone of *cpcot1* for complementation (see text for details). Restriction site *Hind*III* originates from the multicloning site of the vector pBluescript. To check the reinsertion of *cpcot1* for complementation the primers DCOT-hIL1/DCOT2 (B and E) and COTKOMP1/COTKOMP2 (K and L) were used. Abbreviations for restriction enzymes: *BI*, *Bam*HI; *B*, *Bg*II; *EI*, *Eco*RI; *EV*, *Eco*RV; *HIII*, *Hind*III; *K*, *Kpn*I; *P*, *Pst*I; *S*, *Sal*I; *SII*, *Sac*II; and *X*, *Xba*I.

4.5. *CPCOT1* is essential for pathogenicity

To determine to which extent the *cpcot1* deletion has impact on pathogenic development of the fungus, rye florets were inoculated with conidial suspensions of wild-type 20.1 (240 florets), mutants Δ*cpcot1*-1 and -2, (400 florets each), and the complemented strain CC5 (160 florets). The infected plants were daily analyzed for infection symptoms. Whereas in the wild-type and the complemented strain about 7 days post-infection (dpi) droplets of honeydew occurred (the first sign of infection), and after about 2 weeks sclerotia developed (example shown in Fig. 8B), there was no sign of infection in any of the florets infected with mutant spores, indicating full apathogenicity of the mutant strains (Fig. 8C). However, a mutant with an ectopic insertion of the replacement vector showed normal virulence (Fig. 8D).

To pinpoint the block of the infection development, isolated ovaries maintained in axenic culture (see Section 2) were infected with conidial suspensions of the same

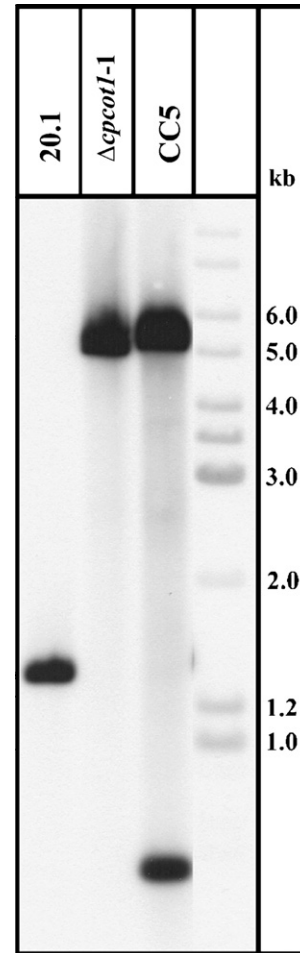


Fig. 4. Southern blot analysis of deletion mutant Δ*cpcot1*-1, complemented strain CC5 and wild-type 20.1. Genomic DNA of selected strains was digested with *Hind*III and *Eco*RI, electrophoresed, blotted, and probed with the right flank of the replacement vector pΔ*cpcot1* (for details see Section 2).

set of strains; 2, 3, 5, and 7dpi the ovaries were cut, stained with aniline blue, and analyzed by fluorescence microscopy.

After inoculation with the wild-type, the well-described infection route (reviewed in Tudzynski and Tenberge, 2003) was observed: 1–2 dpi hyphae were visible within the stigmatic hairs heading for the ovary (Fig. 8H). Three dpi the first hyphal tips started to surround the ovule and after 4–5 days the fungus reached the base of the ovary and started to colonize the entire ovarian tissue by heavily branching. However, in ovaries infected with conidia of the Δ*cpcot1*-mutants no growth could be detected neither in the ovarian tissue nor within the stigmatic hairs. Only a few hyphae could be seen growing on the stigmatic hairs. During growth on the plant's surface the described phenotype of the mutants was visible (Fig. 8I). These findings strongly suggest that the mutants are unable to penetrate the host's tissue. To determine the mutants' ability to grow within the plant we wounded ears before infecting with the mutants. In contrast to

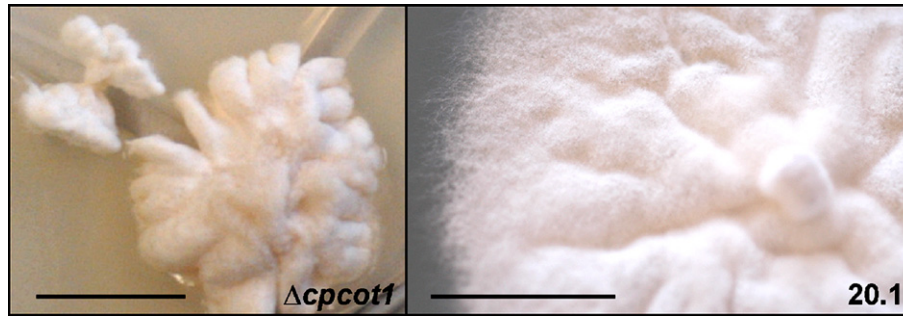


Fig. 5. Axenic growth of the $\Delta cpcot1$ -1 mutant and wild-type strain 20.1. Strains were grown on Mantle medium. Pictures were taken 7 days post-inoculation (scale bar = 1 cm).

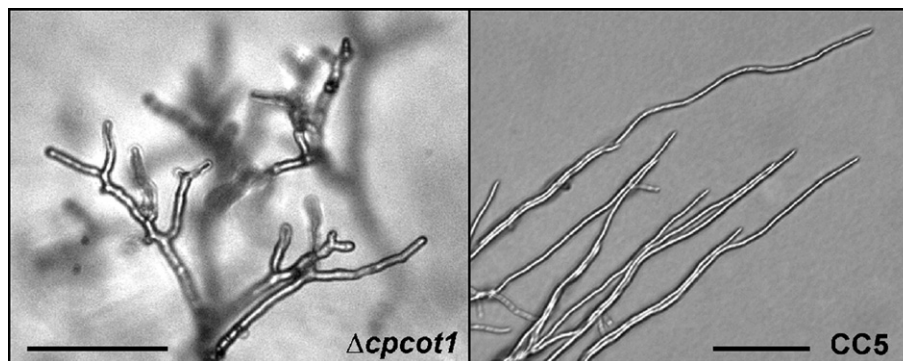


Fig. 6. $\Delta cpcot1$ hyphal morphology. The $\Delta cpcot1$ -1 mutant and the complemented strain CC5 were grown for 4 days on glass slides covered with Mantle medium. The edge of the growing mycelium was directly observed using light microscopy. The scale bar represents 100 μm .

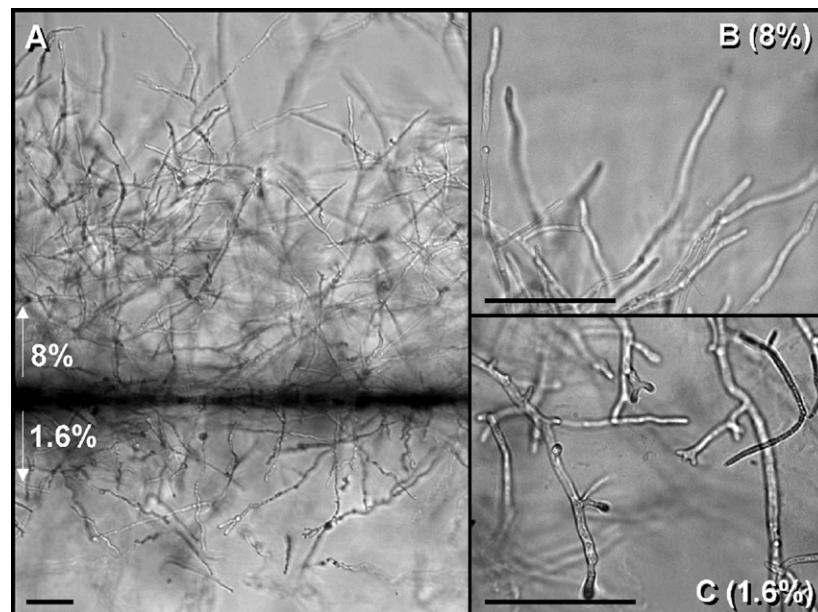


Fig. 7. Invasive growth of the $\Delta cpcot1$ -1 mutant. The mutant was plated on medium containing 1.6% agar and overlaid with 8% low melting agarose. After 3 days thin cross sections were observed using light microscopy (A). (B and C) Details of growth within 8 and 1.6% agar, respectively (scale bar = 100 μm).

wounded ovaries infected with the wild-type (Fig. 8F) no macroscopic sign of infection could be observed (Fig. 8G). Correspondingly, microscopic analyses at five dpi did not show growth within the plant (Fig. 8J). Myce-

lium, which developed on the surface of the wounded tissue, displayed the club-shaped phenotype (Fig. 8K). Restoration of pathogenicity by reinsertion of *cpcot1* was successful: seven dpi conidia-rich honeydew and

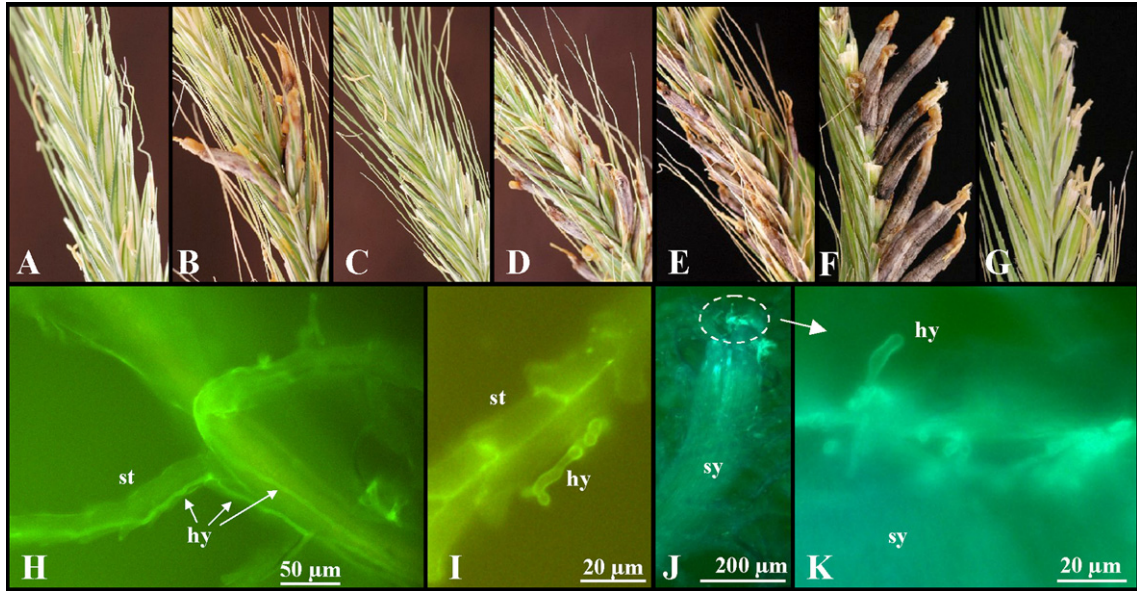


Fig. 8. Effect of the inactivation of *cpcot1* on the ability of *Claviceps purpurea* to penetrate and colonize rye ovaries. Rye florets were infected with (A) water suspension and conidial suspensions from (B) wild-type, (C) mutant $\Delta cpcot1$ -1, (D) a mutant with an ectopic integration and (E) the complemented mutant CC5. Infection of wounded ears was performed by bisection of the florets and infection with conidial suspensions from (F) wild-type and (G) mutant $\Delta cpcot1$ -1. Pictures were taken 4 weeks post-inoculation. Ovaries were infected in vitro with conidial suspensions from (H) wild-type and (I) $\Delta cpcot1$ -1 mutant strains. Additionally, stylar tissue was wounded and inoculated with conidia of $\Delta cpcot1$ -1. Fungal growth is visible in the wounded area (J and K). Stigmatic cell walls and stylar tissue display fluorescent signals due to unspecific staining. All ovaries were collected at 5 days post-inoculation, stained with aniline blue, and observed using epifluorescence microscopy. (hy) hyphae; (st) stigma; and (sy) style.

subsequently sclerotia appeared (Fig. 8E). Ripened sclerotia were transferred to agar plates and observed for mycelial growth. All sclerotia germinated and developed hyphal colonies (data not shown). Thus, the aberrant phenotype of the $\Delta cpcot1$ -mutants is due to the deletion of *cpcot1*.

5. Discussion

The serine/threonine protein kinase CPCOT1, a member of the NDR kinase family has been identified and, for the first time, functionally analyzed in a biotrophic ascomycete—*C. purpurea*. Similar to the complementation of the *N. crassa cot-1* mutant with *tb3*, a homologous gene from *C. trifolii* (Buhr et al., 1996), it could be shown that *cpcot1* is able to complement the *cot-1* mutant. Interestingly, the *C. purpurea* regulatory region of approx. 570 bp seems to be a functional promoter in *N. crassa* (the fact that multiple phenotypically complemented transformants were obtained reduces the likelihood that the *cpcot1*-fragment had integrated close to other active promoters). Earlier studies have shown that heterologous complementation of the *Magnaporthe grisea* $\Delta pmk1$ and $\Delta mps1$ mutants, respectively, with highly conserved MAP kinase genes from *C. purpurea* under their own promoter, is possible (*cpmk1*, *cpmk2*; Mey et al., 2002a,b). As reported here for the complementation of *cot-1*, the wild-type phenotype was fully restored. The

restoration of growth defects in the *N. crassa cot-1* mutant with *cpcot1* indicates that the signal cascade in which COT1 and its homologues are involved is highly conserved, despite the complete difference in their life style—the saprophyte *N. crassa* and the biotrophic phytopathogen *C. purpurea*. Interestingly, sequence analysis of *cpcot1* indicated a possible localization of CPCOT1 in the nucleus due to its glutamine stretch (and a putative nuclear localization signal) as it was found in *C. trifolii* TB3 (Buhr et al., 1996), *U. maydis* UKC1 (Durrenberger and Kronstad, 1999) *S. cerevisiae* Cbk1p (Bidlingmaier et al., 2001) and *warts* (Justice et al., 1995) but not in *N. crassa* (Yarden et al., 1992) although COT1 could also be localized in the nucleus of *N. crassa* (Gorovits et al., 2000).

To further analyze the function of CPCOT1 and its impact on pathogenicity, *cpcot1* deletion strains were constructed. Interestingly, even heterokaryotic transformants carrying transformed and untransformed nuclei were affected in vegetative growth. After single spore isolation this phenotype was more pronounced, indicating that defects caused by deletion of *cpcot1* could not be fully complemented by a heterokaryotic situation. On the other hand, restoration of the wild-type phenotype by reinsertion of *cpcot1* producing a homokaryotic strain with an interrupted and a functional copy of the gene was possible. These results are consistent with a nuclear autonomous function of CPCOT1 as it is also postulated for TB3 (Chen and Dickman, 2002).

The drastic effect on vegetative growth is comparable, in the main features (rounded, short hyphae, hyperbranching, and colonial growth), to the polarity-related defects observed in the *cot-1* mutant of *N. crassa* (Yarden et al., 1992). Detailed pathogenicity tests showed that the $\Delta cpcot1$ mutants were absolutely apathogenic, they were impaired in very early steps of pathogenesis, obvious even during the penetration phase. Our results are in line with the established fact concerning the link between hyphal polarity and pathogenesis, as shown, for example, in the human pathogen *Candida albicans* (Basilana et al., 2003; Zheng et al., 2003).

Since *C. purpurea* does not produce appressoria, other tools for penetration (e.g., cutinases and other cell wall degrading enzymes) could be repressed in the $\Delta cpcot1$ mutant. Mutants defective only in host penetration have been described in *M. grisea*. There, a tetraspanin deficient mutant ($\Delta pls1$) fails to form a penetration peg and is completely apathogenic (Clergeot et al., 2001). However, deletion mutants of signalling chain components impaired in plant infection often show pleiotropic impairment of pathogenicity. For instance, the $\Delta cpmk1$ mutant which represents the deletion of the Pmk1/FUS3 orthologue in *C. purpurea* does not invade the host but is also incapable of growing within the tissue after wounding (Mey et al., 2002a), comparable to the $\Delta cpcot1$ mutants. Obviously, penetration is not the only defect of $\Delta cpcot1$ mutants, since infection experiments with wounded ovaries demonstrated that invasive growth within the plant tissue is also impaired.

Regarding these results (aberrant cell shape, no penetration, and no invasive growth within the plant) it could be concluded that apathogenicity is due to defects in cell wall architecture of the mutants, which make not only penetration but also invasive growth impossible, comparable to $\Delta cpmk2$ mutants (Mey et al., 2002b). Correspondingly, the deletion of *cbk1* in *S. cerevisiae* affects the expression of a range of cell wall-modifying enzymes (Bidlingmaier et al., 2001). Maintenance of localized dynamic balance between cell wall degradation and synthesis is essential for polarized growth. Thus, misregulation of the expression of genes with cell wall-related functions could also cause polarized growth defects as observed in the $\Delta cpcot1$ mutants.

Gorovits and Yarden (2003) demonstrated that the phenotype of the *cot-1* mutant of *N. crassa* is can be suppressed by different external signals. Various environmental stress factors (osmotic and oxidative stress) suppress the *cot-1* phenotype suggesting that the requirement for the fully functional COT1 can be bypassed (Gorovits and Yarden, 2003). In preliminary experiments suppression of the $\Delta cpcot1$ phenotype by amendment of osmotic stress factors as sorbitol or NaCl did not occur. More detailed studies will reveal the connection between external stress factors and CPCOT1 func-

tion. However, partial restoration of the wild-type phenotype in agar invasion tests indicates a possible bypass of the deleted CPCOT1. Thus, a defect in cell wall architecture which can be bypassed seems not to be the only reason for apathogenicity. Thus, CPCOT1 may be linked with environmental sensing, but in a manner which is not identical to that of *N. crassa* COT1 signalling. Different functions of COT1 and Cbk1p—the *S. cerevisiae* homologue—were already postulated by Gorovits and Yarden (2003). This has also been proposed for other highly conserved signalling components exhibiting different functions within different fungi. One example is the function of small GTPases like CDC42, RAS, and RAC, that differs in various fungi like *C. trifolii*, *U. maydis*, and *C. purpurea* (for review see Tudzynski and Scheffer, 2004; Harris and Momany, 2004). Since *C. purpurea* is adapted to its organ specific life style other stress factors—or guidance cues—could be mediated by CPCOT1.

Establishing links to additional components within the signalling network is essential for elucidating the function(s) of CPCOT1. In *N. crassa*, COT1 function is associated with the PKA pathway. Inhibition of PKA in *N. crassa* leads to suppression of the mutant phenotype (Gorovits and Yarden, 2003). The cAMP pathway has been reported to be directly involved in fungal stress response and fungal virulence (reviewed in Lee et al., 2003). As part of an approach to determine if a link exists between CPCOT1 and PKA in this organism, an effort for deleting the adenylyl cyclase of *C. purpurea* (*cpac*) is under way (J. Westendorf, J. Scheffer, P. Tudzynski, unpubl.).

Another linkage of a COT1 homologue has been identified in fission yeast. Orb6p—the homologue of COT1—interacts genetically with the Pak1/Shk1 protein kinase (Verde et al., 1998). CDC15, one of the budding yeast Ste20-like kinases, was identified as the upstream kinase of DBF2, also belonging to the NDR kinase family (Mah et al., 2001). Furthermore, two other STE20-like kinases were placed upstream of the second fission yeast NDR homologue Sid2 (Guertin et al., 2000). Since the hydrophobic motif site (aa 642–651), discussed for being targeted by PAK kinases, is also present in CPCOT1, a linkage between PAK kinases and CPCOT1 is possible. To characterize this interaction in *C. purpurea* the deletion of *cpcla4*, encoding a PAK kinase homologous to *chml* of *M. grisea* (Li et al., 2004) was initiated in our lab (Y. Rolke, P. Tudzynski unpubl.). Interestingly, the phenotype of $\Delta cpcla4$ -mutants is very similar to $\Delta cpcot1$ mutants: a very dense colonial growth structure but with more pronounced morphological features (Y. Rolke, P. Tudzynski unpubl.), in a manner similar to that described by Seiler and Plamann (2003).

The downstream partners of COT1 homologues have yet to be identified; in *C. trifolii* Chen and Dickman

(2002) postulated a direct role of TB3 as a transcriptional regulator. The structural properties of CPCOT1 support such a function: the polyglutamine-rich region in CPCOT1 (aa 43–64) and the nuclear localization signal (aa 223–226) indicate a function of CPCOT1 within the nucleus. On the other hand, CPCOT1 could control a set of downstream factors with transcriptional regulator activity as recently demonstrated in yeast (Schneper et al., 2004). The availability of a $\Delta cpcot1$ mutant allows the identification of potential target genes, either by suppression subtractive hybridization (SSH) or macroarray analyses. It will be especially interesting to see at which point of the very early infection processes of *C. purpurea* the regulatory network of CPCOT1 is essential.

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