A CDC42 Homologue in *Claviceps purpurea* Is Involved in Vegetative Differentiation and Is Essential for Pathogenicity

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*Claviceps purpurea*, a biotrophic pathogen of cereals, has developed a unique pathogenic strategy including an extended period of unbranched directed growth in the host's style and ovarian tissue to tap the vascular system. Since the small GTPase Cdc42 has been shown to be involved in cytoskeleton organization and polarity in other fungi, we investigated the role of Cdc42 in the development and pathogenicity of *C. purpurea*. Expression of heterologous dominant-active (DA) and dominant-negative (DN) alleles of *Colletotrichum trifolii* in a wild strain of *C. purpurea* had significant impact on vegetative differentiation: whereas DA Ctcp42 resulted in loss of conidiation and in aberrant cell shape, expression of DN Ctcp42 stimulated branching and conidiation. Deletion of the endogenous Ctcp42 gene was not lethal but led to a phenotype comparable to that of DN Ctcp42 transformants. ΔCtcp42 mutants were nonpathogenic; i.e., they induced no disease symptoms. Cytological analysis (light microscopy and electron microscopy) revealed that the mutants can penetrate and invade the stylar tissue. However, invasive growth was arrested in an early stage, presumably induced by plant defense reactions (necrosis or increased production of reactive oxygen species), which were never observed in wild-type infection. The data show a significant impact of Ctcp42 on vegetative differentiation and pathogenicity in *C. purpurea*.

Small GTPases are molecular switches that are involved in numerous signal transduction pathways. They mediate signals by switching between active GTP- and inactive GDP-bound states. The entire process is regulated by guanine exchange factors, GTPase-activating proteins, and guanine nucleotide dissociation inhibitors. Guanine exchange factors activate GTPases by catalyzing the exchange of GDP to GTP. The activated GTP-bound form is stimulated by GTPase-activating proteins to hydrolyze GTP. This results in inactivation of the activated GTP-bound form of the GTPase (13, 8). The activated GTP-bound form mediates signals to a variety of downstream effectors. Since the CDC42 Rho GTPase was first identified in a *Saccharomyces cerevisiae* mutant strain and recognized to be necessary for polarized growth and bud emergence (1, 30), it has become increasingly apparent that homologs—which are found in all eukaryotes—are essential for cytoskeletal reorganization and gene expression in response to various signals (20, 24). In *S. cerevisiae*, Cdc42p not only regulates polarized growth and bud emergence but also transduces signals via the p21-activated serine/threonine kinase family members Ste20p (22) and Cla4p (11) to regulate gene expression and septin organization in filamentous growth, mating, and yeast cytokinesis. Existing in two different pools—cytosolic and membrane bound—Cdc42 can function in multiple pathways in the same cell (20).

In contrast to yeast in which cell polarity is mainly required for bud emergence, hyphal growth of filamentous fungi possesses unique features, such as maintaining new axes of polarity and an extremely rapid extension rate (14). These abilities enable them to react to external stimuli and, as a consequence, to colonize their habitats. In the case of phytopathogenic fungi, colonization often requires orientation and directed growth to locate natural entrances (e.g., *Puccinia graminis* [31]) or for orientation within their hosts. A well-described model system for the latter feature is the interaction *Claviceps purpurea*/*rye* (reviewed in reference 46).

*C. purpurea* is a ubiquitous biotrophic ascomycete which specifically colonizes only grass florets. For this purpose, hyphae invade the host at the stigma and follow the pollen tube path to reach vascular tissue at the ovarian base. After securing a stable nutrition supply by tapping the vascular bundles, the fungus colonizes the entire ovary. In this stage *C. purpurea* is able to maintain a continuous flow of phloem exudates for the production of conidia-containing honeydew. To complete pathogenic development, the ovary is replaced by a persisting sclerotium (43, 44). The growth pattern during the first stages of infection, penetration, and directed growth along the pollen tube path differs from that of later stages and from growth in axenic culture since hyphae are mostly unbranched and are clearly guided or attracted by external signals. To obtain a deeper insight into the molecular processes underlying directed growth, we have recently characterized different signal components, such as Cpcot1, a serine/threonine kinase (36) and the p21-activated serine/threonine kinase Cplc44 (Y. Rolke and P. Tudzynski, unpublished). Deletion mutants of both genes were strongly affected in cell morphology/polarity and impaired in penetration and invasive growth.

As polarity is a prerequisite for orientated growth and Cdc42 is a primary switch mediating internal and external stimuli to favor polarized growth, we characterized the impact of
Cdc42 homolog (CtCDC42) was identified and characterized in Colletotrichum trifolii, a filamentous fungal pathogen causing anthracnose disease in alfalfa. Results obtained by genetic approaches revealed that CtCDC42 is involved in spore germination and proper hyphal growth and functions as a negative regulator of appressorium formation (M. Dickman et al., unpublished data).

In this paper we demonstrate the impact of CtCDC42 on hyphal morphology and pathogenicity of C. purpurea. In a first step, we studied the effect of overexpression of constitutive active and negative forms of the highly homologous CtCDC42 gene from C. trifolii. The activation by a glycine-to-valine exchange at position 12 (which corresponds to a G14V in CtCDC42) was originally identified in oncogenic versions of human Ras (41). The mutation causes activation by arresting the protein in a form similar to the GTP-bound conformation (40). Whereas the heterologous expression of the CtCDC42 protein in a form similar to the GTP-bound state (40).

The CtCDC42(T19N) mutation in C. trifolii is equivalent to the cdc42(T17N) mutation which causes an arrest in the GDP-bound state and hence a permanently negative state. Like the dominant negative form, this mutation was first analyzed in human Ras (39). Whereas the heterologous expression of dominant-active (DA) CtCDC42 resulted in loss of conidiation and misshapen bulbous cells, the expression of dominant-negative (DN) CtCDC42 stimulated branching and conidiation. Moreover, we found that deletion of CtCDC42 was possible (i.e., not lethal) and led to a phenotype that was similar to the DN Cdc42 phenotype. DA CtCDC42 expression in the deletion background resulted in the bulbous phenotype. In pathogenicity assays, the lack of CtCDC42 mutants were able to penetrate and invade the host, but invasive growth was arrested in the stylar tissue. Electron microscopic analyses detected elevated levels of reactive oxygen species (ROS) in the apoplastic of the colonized plant tissue, and necrotic reactions were induced. The data presented here suggest a strong impact of CtCDC42 not only on hyphal morphology but also on host pathogen interaction.

**Materials and Methods**

**Strains, 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 (19). For conidia harvesting and DNA isolation, mycelia were cultivated on Mantle agar (16 g/liter of agar) with 100 μl of sucrose (23) at 28°C for 12 to 14 days. 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.

**Nucleic acid extraction and analysis.** Standard recombinant DNA methods were performed according to Sambrook et al. (34) andAusubel et al. (4). Genomic DNA from C. purpurea was prepared from hyphalized mycelium according to the method of Cenis (7). For Southern blot analysis, 5 to 10 μg of restriction-digested chromosomal DNA or PCR products was electrophoresed in 0.8 to 1.6% agarose gels with salt-free buffer (34), blotted onto positively charged nylon filters (Hybond N+; Amersham, Braunschweig, Germany), and hybridized to radioactivity-labeled DNA probes in Denhardt's hybridization solution (34). 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 and for 10 min in 1× SSC-0.1% sodium dodecyl sulfate. 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. (25). Protein and DNA sequence alignment, editing, and organization were done with DNA Star (Madison, WI). Sequence analysis was done using BLAST at the National Center for Biotechnology Information, Bethesda, Md. (2). PCR was done as described by Sambrook and colleagues (34), using Red Taq Polymerase (Sigma, Milwaukee, WI). All primers were synthesized by MWG-Biotech (München, Germany). The amplification products were cloned with a PCR 2.1 TOPO-Cloning Kit from Invitrogen.

**Cloning of CtCDC42 and generation of a replacement vector.** For the amplification of an internal fragment of CtCDC42, degenerate primers were designed using the CODEHOP program (http://blocks.fhcrc.org/blocks/codehop.html) (33) and the amino acid sequences of Cdc42 homologs of Aaspergillus nidulans (AAB24513.), S. cerevisiae (P12073), Schizosaccharomyces pombe (Q01112), Ashbya gossypii (AAG14271), Glomerella cingulata (AAD00177), and Sollias bosvianum (AAC37871). With the deduced primers cdc42-3 (5'-GAC TAC GTC CCC ACC GTC TTY GAY AAY TA-3') and cdc42-4 (5'-GGG CGG AGC ACT CGA CAY AYT TXA C-3'), a fragment of 495 bp was amplified showing high homology to Cdc42 in various fungi. With this fragment as a probe, we screened a genomic library of strain T5 (38) by plaque filter hybridization (34). From the 34,000 lambda clones screened, 10 hybridized to the PCR probe and 4 of them were contained an overlapping fragment. The overlapping and Southern blot analyses. From phage number 3 a 4.1-kb KpnI fragment was cloned into pUC19 resulting in pCDC2K1. This clone, containing the 1.0-kb open reading frame together with a 0.96-kb 5′ sequence and a 2.1-kb 3′ sequence, was subcloned and sequenced. Restriction analyses and sequencing revealed another KpnI site close to the 3′ end of pCDC2K1 due to partial restriction of phage number 3. For the construction of the pCDC2K1 replacement vector, the genomic regions upstream and downstream of the cdc42 fragment (see Fig. 3) were amplified with 10 μg of the pApcdc42 plasmid. The linear replacement construct was excised using NotI-KpnI and subsequently used to transform the C. purpurea wild-type strain 20.1 (see Fig. 3A). The vector for the complementation of ΔCtCDC42 mutants was obtained by cloning a 3.9-kb fragment from pCDC2K1 into the corresponding restriction site of the pA7N1-UM (26) vector, producing the pApcdc42 plasmid. The linear replacement construct was excised using NotI-KpnI and subsequently used to transform the C. purpurea wild-type strain 20.1 (see Fig. 3A). The vector for the complementation of ΔCtCDC42 mutants was obtained by cloning a 3.9-kb fragment from pCDC2K1 into the corresponding restriction site of the pA7N1-UM (26) vector (see Fig. 3A). This vector was termed pCdc42 and was used to transform the ΔCtCDC42 mutant strain ΔCtCDC42-1.

**Fungal transformation.** Protoplasts of C. purpurea generated with lysing enzymes from Trichoderma harzianum and Driselase (InterSpex) were transformed with 10 μg of the pApcdc42 fragment (see Fig. 3) as described by Jungehülsing et al. (21). For hygromycin selection, protoplasts were incubated at 28°C for 24 h, after which they were overlaid with 10 ml of 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. Resistant colonies were transferred to fresh selective medium (BII, pH 8, containing 0.5 mg/ml hygromycin) and screened for homologous integration by PCR. To obtain homokaryotic strains, the transformants were subjected to at least one round of single spore isolation. Primer pairs DCD-clF1/CDCLF2 and DCD-clR1/CDCLIR2 were used to identify transformants with a homologous integration of the 5′ flank and 3′ flank, respectively (see Fig. 3A). The predicted 1,664- and 2,350-bp fragments could be amplified with two strains, termed ΔCtCDC42-1 and ΔCtCDC42-2. The lack of the wild-type gene copy in the ΔCtCDC42 mutants was checked using the primer DCD-clF1/DCD-clR1 (5′-GGC GAC ACC TTC CCT ACT CGA CAY AYT TXA C-3′) and DCD-clR2 (5′-CGG CAT CTT ATC GCC TTC TTC CT-3′) which gave rise to a 1,279-bp fragment in the wild-type strain. Complementation and insertion of heterologous genes were done by transformation of circular plasmids carrying the phleomycin resistance gene (ble) as a selectable marker. For phleomycin selection, phleomycin was directly applied to the protoplasts to a
final concentration of 33 μg/ml of modified BII medium (pH 8.2, 20% sucrose and no FeSO₄). Resistant colonies were transferred to fresh selective medium (BII, pH 8.2, containing 100 μg/ml of phleomycin) and subjected to at least one round of single-spore isolation to obtain homokaryotic transformed strains. In the case of nonsporulating transformants, hyphal tips grown on selective medium were isolated and placed on fresh selective medium. Growing colonies were transferred to nonselective medium and finally to selective medium to confirm stable integration. Reintegration of the complete Cdc42 sequence including the promoter region of about 900 bp was determined by PCR with primers DCDC-hIL1 (5′-GGT TTA-3′)/H11032-5′-CGA AGT GGA AAG GCT GGT GTG-3′; CTG CCA GCA AAA GAC AAG AAA GAA-3′) and CTG CCA GCA AAA GAC AAG AAA GAA-3′). PCR fragments were cloned into the Cdc42 alleles (see text) are indicated by arrows. The known functional domains are indicated as follows: dashed box, GTP binding/hydrolysis domains; bold black box, effector domain; dotted box, Rho insert domain; and thin black box, membrane localization domain.

RESULTS
Cdc42 encodes a highly conserved Cdc42 homolog. Using the CODEHOP program, we designed two degenerated primers (Cdc42-1 and Cdc42-2) based on a theory of primer design in which the degree of degeneration is minimized using an invariant core region at the 5′ end and a variable clamp region at the 3′ end (33). The derived amino acid sequence of the PCR fragment showed high homology to the Cdc42-like proteins CflA of Penicillium marneffei (AAK56917) and MgCdc42 of Magnaporthe grisea (AAF73431), and this fragment was used to screen an EMBL3 genomic library of C. purpurea. By Southern analyses (see Fig. 3B), we identified, encoding a polypeptide of about 22 kDa (Fig. 1). The derived amino acid sequence is 92%
identical to Cdc42 and shows high homology to several Cdc42 homologs (Fig. 1). The sequence contains all domains previously shown to be necessary in Cdc42 function of S. cerevisiae, S. pombe, and others (for review, see reference 20). These domains include four GTP binding and hydrolysis domains, an effector domain, a Rho insert domain, and a membrane localization domain (see Fig. 1). Therefore, the gene was termed Cpcdc42. Southern blot analyses under low-stringency conditions, using digested genomic DNA from the wild-type strain 20.1 and the PCR product used for the screening of the genomic library as a probe, confirmed that Cpcdc42 is a single copy gene (data not shown).

**Heterologous expression of dominant active Cdc42(G14V) and dominant negative Cdc42(T19N) in C. purpurea.** Since deletion of Cdc42 was found to be lethal in many eukaryotes (20), we used an alternative approach to characterize CpCDC42 by expressing dominant active and dominant negative forms of this protein. Based on the significantly high similarity between Cpcdc42 and Ccd42, we transformed C. purpurea wild-type strain 20.1 with several Ccd42 constructs, including the DA Ccd42(G14V), the DN Ccd42(T19N) and—as a control—the wild-type Ccd42, all under the control of the constitutive gpdA promoter (32). This promoter was previously shown to be functional in C. purpurea (e.g., reference 48). For simplicity, the obtained mutants were named DN, DA, and DCtWT, respectively (Table 1). Phleomycin-resistant transformants were checked by PCR for the integration of the complete promoter-gene constructs. For each DNA construct, four independent fungal strains were further analyzed. To separate transformed from nontransformed nuclei of possible heterokaryotic strains, one round of single spore isolation or—with strains impaired in conidiation—iso lation of hyphal tips grown on selective medium was performed. One passage of growth on nonselective medium followed by one on selective medium was done to confirm stable integration of the transgenes. All strains bearing the same DNA construct were phenotypically similar. This observation together with the severity of the phenotypes represents sufficient evidence that the transgenes were expressed in the transformants.

*C. purpurea* is able to grow in axenic culture on solid and liquid media. On rich solid medium such as Mantle medium (23), a growth rate of about 2 to 3 mm/day is reached. Under these conditions conidiation starts after 7 days. Grown in axenic culture on solid Mantle medium, the transformants carrying the wild-type Ctcdc42 (DCtWT) showed no differences in germination, hyphal development, and growth rate. Conidiation was unaffected and comparable to the wild-type 20.1 (Fig. 2C). However, both the DA Ctcdc42 strains and the DN Ctcdc42 strains were drastically altered in growth behavior. DA strains revealed extremely distorted growth: formation of hyphae was unequal due to the appearance of extremely enlarged vacuoles (Fig. 2A). Conidiation was never observed in these strains. This growth behavior resembles the bulbous storage cells in the sclerotal stage of in planta infection (43). In addition, after 7 days in axenic culture, autolysis started from the center of the colony (data not shown). The DN transformants had a completely different phenotype: they showed enhanced branching along with massive production of conidia. Conidiation already appeared after 2 days; germination of conidia was not affected. In contrast to the DA phenotype, proportion of the hyphae did not differ from wild type (Fig. 2B). To further analyze these transformants, we performed pathogenicity assays. As a first indication of successful infection, the period of time until appearance of honeydew, a sugar-rich fluid serving as distributor of conidia, is measured. Inoculation with *C. purpurea* wild-type 20.1 leads to honeydew formation after 6 to 7 days postinoculation (dpi). About 14 dpi the development of sclerotia is initiated. For pathogenicity tests, rye florets were inoculated with conidial suspensions of

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### TABLE 1. Overview of CDC42-transformation experiments

| Strain | Construct $^b$ | Morphological phenotype | Pathogenicity $^c$ |
|--------|----------------|-------------------------|------------------|
| DN     | Wild type     | Oe; DN Ctcdc42         | Enhanced branching, increased conidiation | + |
| DA     | Wild type     | Oe; DA Ctcdc42         | Bulbous hyphae, no conidia              | (+) |
| DCtWT  | Wild type     | Oe; WT Ctcdc42         | Comparable to wild type                  | + |
| Δ      | ΔCpcdc42      | ΔCpcdc42                | Like DN, but more pronounced              | − |
| ΔDA    | ΔCpcdc42      | ΔCpcdc42                | Like DA, but more pronounced              | − |
| ΔDCtWT | ΔCpcdc42      | ΔCpcdc42                | Restoration of wild type                  | + |
| ΔCpWT  | ΔCpcdc42      | CpCDC42                | Restoration of wild type                  | + |

$^a$ For simplicity, these abbreviations are used to describe the mutants in the text.

$^b$ Abbreviations: Oe, overexpression; DA, dominant active; DN, dominant negative; WT, wild-type; CpCDC42, CDC42 homologue of *C. purpurea*; Ctcdc42, CDC42 homologue of *C. infuli*.

$^c$ +, fully pathogenic on rye, including honeydew production and sclerotia formation; (+), limited infection rate; −, apathogenic.
DN and DCtWT strains and, as a control, wild-type strain 20.1. Inoculation with the nonsporulating DA strain was carried out by using suspensions of mycelium, together with equally treated wild-type strain 20.1. For each strain, 150 rye ears were infected. As a control for viability, the conidial or mycelial suspensions that were used for infection were plated on solid medium. All strains germinated and grew in axenic culture; only the DA strains showed reduced viability, probably due to altered cell architecture (see above). Surprisingly, neither the DCtWT transformants nor the DN transformants showed a distinct alteration in virulence. Time course of honeydew and sclerotia formation was equal to that of the wild type. In contrast, most of the tested DA strains did not infect at all; only the infection with one strain (DA3) led to a (retarded) production of honeydew (10 to 12 dpi) in two out of five ears. Mycelium regenerated from honeydew of these ears still showed phleomycin resistance but had a rather variable phenotype, indicating a heterokaryotic status of this transformant (data not shown).

**Generation of ΔCpcdc42 mutants.** Although the DN strains showed drastic morphological alterations in axenic growth, no reduction of pathogenicity could be observed, possibly due to the presence of the Cpcdc42 wild-type gene. Therefore, we implemented a gene replacement approach. As outlined in Fig. 3A, S' and 3' parts of Cpcdc42 on each side of a hygromycin resistance cassette (tpph) in the pAN7-1UM plasmid (see Material and Methods for details). The resistance cassette, excised using a NotI-KpnI restriction, was used to transform the C. purpurea wild-type strain 20.1. Δcpcdc42 mutants were generated following the disruption of the wild-type gene (WT) by homologous recombination through a double crossover event. The Cpcdc42 coding sequence and introns are represented with black and white boxes, respectively. The ATG indicates the start of translation of Cpcdc42, and the black arrow indicates the orientation of the hygromycin resistance cassette within the replacement construct. Primers DCDCCLF1/DCDCCLF2 (a and b) and DCDCCRF1/DCDCRF2 (c and d) used for the amplification of the upstream and downstream flanks, respectively, are indicated by black arrows. The positions of primers DCDC-hIL1/DCDC-hIL2 (e and f) and DCDC-hIR1/DCDC-hIR2 (g and h), used for the identification of homologous integrations, and primers DCDC-WT1/DCDC-WT2 (i and j), used for detection of the Cpcdc42 wild-type copy, are indicated with black triangles. The DNA fragment used as a probe in the Southern blot shown in panel B is indicated as a striped box. The full-length clone of Cpcdc42 for complementation is shown (see text for details). To check the reinsertion of Cpcdc42 for complementation, the primers DCDC-hIL1/CCDC42-1 (c and k) were used. Abbreviations for restriction enzymes: Cl, ClaI; EV, EcoRV, KI, KpnI; NI, NotI; SI, SacI; XI, XbaI. (B) Southern analysis of deletion mutants ΔCpcdc42-1, ΔCpcdc42-2, complemented strain Ccpcdc42-1, and wild-type 20.1. Genomic DNA of selected strains was digested with ClaI, separated in an agarose gel, blotted, and probed with the right flank of the replacement vector pApcpcdc42. A successful deletion by gene replacement is demonstrated by the shift of the wild-type band (2.6 kb) to 5.75 kb in the lanes of ΔCpcdc42-1 and ΔCpcdc42-2. The complementation leads to the reappearance of the 2.6-kb band and an additional band (0.64 kb). For restriction sites and location of the probe, see panel A.
are characterized by a shift of the wt band (2.6 kb) to the homologous integration band (5.75 kb). The knockout strains were termed \( \Delta \text{H9004} \text{Cp}_4 \text{cdc42-1} \) and \( \Delta \text{H9004} \text{Cp}_4 \text{cdc42-2} \).

\textbf{Cp\textsubscript{cdc42} has impact on conidiation and branching.} The \( \Delta \text{Cp\textsubscript{cdc42}} \) mutants showed drastic phenotypes in axenic culture. Already 2 days after inoculation on solid medium, massive conidiation was initiated. This phenomenon was compared to the wild type in detail. In axenic culture the wild type first colonizes the medium by forming long hyphae with rare branches (Fig. 2D), and later phialidic conidia arranged in heads called conidiophores start to form (characterized in detail by reference 29). In contrast, the \( \Delta \text{Cp\textsubscript{cdc42}} \) mutants differ in terms of conidiation in two aspects: (i) branching was very early and mainly to form conidiophores and (ii) the occurrence

\textbf{FIG. 4.} Axenic growth of the \( \Delta \text{Cp\textsubscript{cdc42}-1} \) mutant (A to C), complemented mutant \( \text{Cp\textsubscript{cdc42}-1} \) (D and E), \( \Delta \text{Cp\textsubscript{cdc42}} \) mutant overexpressing \( \text{DA Ctc\textsubscript{cdc42}}(\text{G14V}) \) (F to I), and \( \Delta \text{Cp\textsubscript{cdc42}} \) mutant overexpressing wild-type \( \text{Ctc\textsubscript{cdc42}} \) (\( \Delta \text{Cp\textsubscript{cdc42}-WT Ctc\textsubscript{cdc42}-1} \)) (J to L). For \( \Delta \text{Cp\textsubscript{cdc42}-1} \) young mycelium with phialidic branches are shown in panels A and B in detail. Massive conidiation occurs after 2 days (C). In the complemented strain \( \text{Cp\textsubscript{cdc42}-1} \), complementation restored hyphal morphology, as shown in panel D (E, detailed view). For \( \Delta \text{Cp\textsubscript{cdc42}-DA Ctc\textsubscript{cdc42}-1} \), swollen hyphae (F to H) and bulbous hyphal tips are shown (I). In \( \Delta \text{Cp\textsubscript{cdc42}-WT Ctc\textsubscript{cdc42}-1} \), hyphal morphology is restored (J and K). Occasionally, an unusual branching pattern was observed (L), which, however, did not form conidia as did \( \Delta \text{Cp\textsubscript{cdc42}-1} \). Compare wild type with Fig. 2D. Strains were grown on Mantle medium. Pictures were taken 5 dpi. Scale bars, 10 \( \mu \text{m} \).

\textbf{FIG. 5.} Pathogenicity assays of \( \text{C. purpurea} \) transformants on rye. Rye florets were infected with water (A) and conidial suspensions from wild type (B), \( \Delta \text{Cp\textsubscript{cdc42}-1} \) mutant (C), the complemented strain \( \text{Cp\textsubscript{cdc42}-1} \) (D), a \( \Delta \text{Cp\textsubscript{cdc42}} \) mutant overexpressing wild-type \( \text{Ctc\textsubscript{cdc42}} \) (E), and a \( \Delta \text{Cp\textsubscript{cdc42}} \) mutant overexpressing \( \text{Ctc\textsubscript{cdc42}}(\text{G14V}) \) (F). Pictures were taken 4 weeks postinoculation. Arrows indicate sclerotia.
of conidiophores was considerably more frequent (Fig. 4A to C). The germination rates of conidia and the size and proportions of hyphae did not differ from the wild type. This phenotype in axenic culture corresponds to that of the DN form but is much more pronounced.

Cpcdc42 is essential for pathogenicity. To test if the deletion of Cpcdc42 alters the virulence of C. purpurea, conidial suspensions of the two mutant strains and the wild-type strain of Cpcdc42 could be detected in the basal part of the stylar tissue and the vascular bundles and starts to colonize the whole ovarian tissue toward the basal part of the ovary where the fungus taps the enter the ovarian transmitting tissue, followed by the growth of the first 2 dpi. Three to five days postinfection, hyphae type germinates and penetrates the plant’s stigmatic hairs during microscopic observation. Normally different periods of time. After cutting and staining (for details infected in vitro cultivated rye ovaries and incubated them for microscopic analyses were necessary. For this purpose we in-

FIG. 6. Effect of the inactivation of Cpcdc42 on pathogenicity of C. purpurea. Ovaries were infected in vitro with conidial suspensions from wild type (A, D, and F) and ΔCpcdc42-1 mutant (B, C, E, and H). A schematic overview of a rye ovary is given in panel G. The solid-lined box represents details shown in pictures panels A to E, while the dotted box represents pictures in panels F and H. Hyphal growth within the stigmas and stigmatic hairs is visible in both the wild-type (A and D) and the ΔCpcdc42-1 mutant (B, C, and E). In contrast to the massive colonization of the transmitting tissue by the wild type (F), no hyphae could be detected in this area after infection with the ΔCpcdc42-1 mutant. Ovaries were collected at 5 days postinoculation, stained with aniline blue, and observed using epifluorescence micros-copy. Arrows indicate hyphae.

Absence of Cpcdc42 triggers H$_2$O$_2$ production in planta. Using ultrathin sections treated with cerium chloride, H$_2$O$_2$ production was detectable in early stages of subcuticular growth of the wild type (visible as an electron-dense precipitate of cerium perhydroxide); the peroxide seems to stem from both interaction partners (Fig. 7D and E). This is the first time that ROS production has been demonstrated in rye tissue infected by a wild strain of C. purpurea; analyses of later infection stages had indicated that the fungus is not recognized by the plant (see reference 27). Accordingly, in the subepidermal stylar tissue, no signal could be detected, indicating that the wild type represses (or does not induce) H$_2$O$_2$ generation in this stage of infection; both the plant tissue and the hyphae look healthy (Fig. 7F). In contrast, during infection with the ΔCpcdc42 mutant H$_2$O$_2$ occurs in high concentration also in subepidermal tissue, probably stemming from both partners (Fig. 7I). Additionally, a breakdown of plant cell compart-
ments can be observed (Fig. 7J). This degeneration of plant cells is accompanied by the occurrence of granular electron-dense particles (phenolic/tannic substances?), as is visible in non-CeCl$_2$ treated samples (Fig. 7K).

Complementation of ΔCpcdc42 mutants. To prove that the described phenotype of the ΔCpcdc42 mutants was due to the deletion of Cpcdc42, we complemented a ΔCpcdc42 mutant by ectopic integration of the complete gene, including the pro-
moter region of about 1 kb (see Material and Methods). Phleomycin-resistant strains were checked for complete integration of the gene by PCR and Southern analyses (Fig. 3B). It was demonstrated that in these strains the phenotype in axenic culture and the pathogenicity on rye were restored (Fig. 4D and E and 5D, respectively).

Heterologous expression of Ctdc42 in a ΔCpcdc42 background restores wild-type hyphal morphology and pathogenicity. Since in axenic culture the phenotype of the ΔCpcdc42 mutants is very similar to that of transformants carrying the DN form of Ctdc42, we wanted to know if a heterologous complementation with Ctdc42 is possible. We introduced the wild-type Ctdc42 under the control of the gpdA promoter into a ΔCpcdc42 mutant using the same construct as described above. Transformants were purified by single spore isolation and evaluated by PCR for complete integration of the pro-
moter and Ctdc42. Five out of eight strains analyzed were similar in phenotype to C. purpurea wild type, and conidiation was reduced to the normal extent. However, hyphae tended to
branch more often than in the wild type (Fig. 4L). To test if this heterologous complementation could also restore pathogenicity, rye ears were infected with conidia of strains showing restored growth characteristics (see above). In two of the tested strains, pathogenicity was fully restored (Fig. 5E): production of honeydew was comparable to the wild type. To ensure that this restoration of pathogenicity was not due to cross-contamination with wild type, we analyzed mycelia derived from honeydew; they were still hygromycin and phleomycin resistant. In addition, the presence of Ctcpdc42 could be demonstrated by PCR (data not shown). The successful complementation of the ΔCpdc42 mutant by the heterologous gene proves that Ctcdc42 is functional in C. purpurea.

**Heterologous expression of the dominant active Ctcpdc42 (G14V) allele in a ΔCpdc42 background.** The effect of expression of the DA Ctcpdc42 in C. purpurea wild type (Table 1, DA) on pathogenicity was not uniform in all transformants tested (see above). Therefore, expression of the DA form of Ctcpdc42 in the ΔCpdc42 background was analyzed. For introduction of DA Ctcpdc42 in a ΔCpdc42 mutant, we used the same DA

![FIG. 7. Light and electron microscopic analyses of rye stylar tissues infected by wild type and ΔCpdc42. (A and B) longitudinal section of a style colonized by wild-type C. purpurea showing mostly intercellular growth between the prosenchymatic host tissue. (C) Rye stylar tissue inoculated with ΔCpdc42. The transition from normal-looking, highly vacuolated host cells to an area of obviously collapsed host cells showing dense staining of the whole cell compartment is visible. (D to F and I to K) In situ detection of H2O2 using the CeCl3 technique. Electron-dense precipitate of ceriumperhydroxide represents the areas where H2O2 was formed. (D) Subcuticular wild-type hypha which shows cell wall-bound and secreted H2O2. Note that the host cell completely lacks any signs of H2O2 generation. (E) Wild-type hyphae, growing both epicuticular (asterisk) and subcuticular, showing intense formation of H2O2. Note also that the host epidermal cells produce H2O2 at this interaction site. (F) Wild-type hyphae among the prosenchymatic transmitting tissue. In this tissue virtually no generation of H2O2 takes place. (G) A ΔCpdc42 hypha inside the stigmatic trichomes. (H) Sparse colonization of stylar tissue by ΔCpdc42 hyphae (arrow). (I) H2O2 production in noncollapsed prosenchymatic host tissue (as depicted in the upper part of panel C adjacent to ΔCpdc42 hyphae). Strong signals can be found in the area of the plasmalemma, vacuoles, and host cell wall (arrow). The host cells show clear signs of breakdown of cell compartments like disintegration of the vacuolar system. (J) H2O2 detection in collapsed host tissue (as depicted in the lower part of panel C). A plasma membrane-bound signal is visible. (K) Collapsed host tissue (CeCl3 treatment was omitted). Granular electron-dense particles are visible in large parts of the cell compartments pointing to the occurrence of phenolic/tannic substances in this area. (L) Noncollapsed host tissue (CeCl3 treatment was omitted). No electron-dense precipitation structures are detectable. Toluidine blue (pH 6.8) staining was used in panels A to C, G, and H. Scale bars: 150 μm (A and C), 10 μm (B, G, and H), 5 μm (D, E, and J to L), and 2 μm (F and I). f, fungus; hc, host cell.
construct as for the transformation of the wild type. The correct integration of the construct was determined by PCR. After one round of hyphal tip isolation followed by growth on selective medium, four independent transformants were characterized. They showed a similar phenotype as the DA transformants of the wild type: swollen vacuoles and an aberrant cell shape and complete suppression of conidiation (Fig. 4F to I). In comparison to the DA form in wild-type background, however, this phenotype was much more severe, and segments of normally shaped hyphae were not detected. In pathogenicity assays (with mycelial suspensions, 150 florets per strain) no symptoms of infection could be detected (Fig. 5F). However, analysis of the mycelial suspension used for infection on agar plates showed reduced viability. Therefore, the observed (in this case uniform) nonpathogenicity of the transformants could be mainly due to reduced fitness.

**DISCUSSION**

In this study we describe characterization of the Cpcdc42 gene encoding a highly conserved Cdc42p-like protein using two strategies: (i) the overexpression of constitutive active and negative forms of Ctcdc42, a Cdc42 ortholog from *C. trifolii* and (ii) the deletion of Cpcdc42.

As a prerequisite for using dominant active and negative alleles of the heterologous Cdc42 gene, the heterologous constructs were proved to be functional in *C. purpurea*: (i) the overexpression of wild-type Ctcdc42 in *C. purpurea* wild-type background did not lead to an impaired phenotype with respect to vegetative growth and pathogenicity, (ii) the dominant-negative construct induced the same phenotype—albeit less severe—as the deletion of Cpcdc42, and (iii) a △Cpcdc42 mutant could be complemented by the heterologous expression of wild-type Ctcdc42. As shown here and in several previous studies (e.g., references 6 and 47), the use of a constitutive promoter instead of the original promoter did not affect the function of the GTPase. Thus, increased concentration of the protein does not alter the ability to transduce signals as long as the proper function is secured. However, if the original protein competes with a gene product of an additionally introduced gene, a competitive situation is created and the proportion of the two proteins is decisive. As described above, the phenotype of transformants of the wild-type strain expressing the dominant negative Ctcdc42(T19N) was less severe than the effect of deleting Cpcdc42. In addition, the effect of the DA form in a △Cpcdc42 background was more pronounced than in the wild-type background.

Nevertheless, heterologous expression of the DA and DN constructs resulted in significant phenotypic alterations. The DA transformants showed misshapen hyphae: cells were swollen and had a spherical rather than elongated shape, autolysis started prematurely, and conidiation was not observed. These findings partially parallel results in other fungi. The activation of homologs of Cdc42 in *Wangiella dermatitidis* and *P. marneffei* resulted in swollen hyphae very similar to the DA transformants of *C. purpurea* (6, 50). In contrast, a lethal phenotype was observed by introducing the dominant activated form into *Candida albicans* (47) and *Saccharomyces cerevisiae* (51). Considering the similarity in cell deformation in DA transformants of *C. purpurea* to the phenotypes of *W. dermatitidis* and *P. marneffei* DA strains, it can be assumed that the dominant activation in *C. purpurea* also leads to a similar deregulation of cell polarity via actin cytoskeleton organization. Further investigations of this phenotype are necessary to verify this hypothesis.

Regarding the impact on pathogenicity, a partial reduction of virulence in the DA strains with wild-type background and a complete loss in the DA strains with △Cpcdc42 background could be due to the defect in cell architecture caused by the influence of activated Cdc42. This instability could prevent proper growth and penetration. Since mycelial suspensions used for infection hardly regenerated after plating, it is not surprising that hyphae of these strains do not possess the power to invade the host. Therefore, concerning pathogenicity, attention should rather be focused on transformants with dominant-negative and null Cdc42, respectively.

As mentioned above, the phenotypes of the DN strains with wild-type background and the △Cpcdc42 mutants were similar, but in the △Cpcdc42 mutants the effect was much more pronounced. Both strains were viable. Hyphal morphology was not affected in terms of hyphal shape, colony growth, and germ tube emergence. However, a higher frequency of branching combined with an enormous increase of conidiation occurred. To our knowledge this morphological phenotype was never described before. In contrast, the heterologous expression in *A. nidulans* of both the DA (G14V) and the DN (D120A) version of CflA, which is a homolog to Cdc42 in *P. marneffei*, eliminated conidiation completely. It was concluded that proper Cdc42 function is required for conidiation in *A. nidulans*, which is not the case in *P. marneffei* (6). Given the full restoration of wild-type phenotype in a △Cpcdc42 mutant by the heterologous Ctcdc42 gene, it is interesting that the effects of the DA and DN alleles in the two fungi are not identical. In *C. trifolii*, the effect of DA Ctcdc42 expression is observed only in minimal medium (and can be reversed by addition of proline); transformants show heavily distorted hyphal growth and lack of appressoria formation, similar to the dominant-active Ras mutant (M. Dickman, et al., unpublished). In contrast to *C. purpurea*, DN Ctcdc42 transformants show dramatically reduced conidial germination. In addition, they display increased appressoria formation under noninducible conditions such as soft agar surface, suggesting that Ctcdc42 is a negative regulator of appressoria formation (M. Dickman et al., unpublished).

Infection assays on rye with the △cdc42 mutants revealed a loss of pathogenicity: symptoms of infection (honeydew and sclerotia) were completely absent. This observation was remarkable since hyphal growth of the mutants obviously was not impaired, and the mutants were still able to invade the host. Microscopic analyses demonstrated that after germination, hyphae directly penetrate the stigmatic hairs. Additionally, we could observe hyphae within the stigmas following the wild-type infection path. As hyphal growth of the △cdc42 strains never occurred within the ovarian tissue, even after a prolonged period of time, fungal growth stops in the basal part of the style. Until this infection stage the fungus has to overcome different barriers within its host, such as penetrating the cuticle which covers the epidermis of the stigmatic cells, growing between epidermal cell walls, orientating within the host’s apoplast (reviewed in reference 45). Since the △cdc42 mutants can
cope with all these barriers in a period of time comparable to that of wild-type infection, the mutants seem to face new aspects of resistance in the transmitting tissue. Light- and electron microscopic analyses showed that, in contrast to the wild type, during infection with the ΔCpcdc42 mutant the stylar cells became necrotic; a comparable degeneration of styal tissue has been observed after successful pollination, obviously in order to prevent competition of multiple pollen tubes (15). Thus, the ΔCpcdc42 mutant evokes a necrotic reaction that obviously prevents further progress of infection. This plant defense reaction and detection of H₂O₂ was unexpected, since the primary function of cdc42 generally involves the cytoskeleton.

The role of ROS in the C. purpurea/rye interaction has been studied by our group in detail (reviewed in reference 46). Cytological analyses of wild-type-infected ovarian tissue has never detected significant levels of ROS formation, indicating that the biotroph C. purpurea effectively hides itself and is not recognized by the plant; i.e., it provokes no defense reactions. In this study we can show for the first time a plant reaction. The epidermal cells produce H₂O₂, but after penetration and during growth in the center of the style, no further reactions are detectable. Recently we showed that deletion of the gene cptf1, encoding a Bzip-transcription factor in C. purpurea, led to a drastic reduction of activity of all known catalase isozymes (27). In addition, the mutants showed significantly reduced virulence. In contrast to the wild type, infection by the Δcptf1 mutants triggered an oxidative burst during colonization. Interestingly, the fungus itself also secreted H₂O₂ in significant amounts. It was postulated that the down-regulation of catalase activity in the Δcptf1 mutants reduces the decomposition of H₂O₂ secreted by the fungus. This could cause a host defense reaction, which is similar to that caused by the ΔCpcdc42 mutants, but occurs in later stages (in ovarian tissue). It will be interesting to reveal the molecular background for the effect of Cpcdc42 on the ROS status in plants. Preliminary northern analyses showed that the expression of a CPTF1-target gene, the catalase gene Cpca1l, is not affected by the Cpcdc42 deletion; i.e., the similar effect of the ΔCpcdc42 and Δcptf1 mutants is not due to a direct effect of CpcDC42 on the transcription factor. If the observed oxidative-burst-like phenotype is caused by increased H₂O₂ secretion by the fungus (as postulated for Δcptf1 [27]), this could also be due to an increased ROS production, e.g., by enhanced levels of O₂⁻ produced by an NADPH oxidase (Nox). Nox activity and ROS production have been shown to be controlled by a second small GTPase, Rac, in mammalian systems and also in a filamentous fungus (9). Recently it could be shown that Cdc42 interacts antagonistically with Rac in control of O₂⁻ production: Cdc42 can act as an inhibitor of the Rac-mediated activation of Nox in mammalian cells (12); our finding of increased ROS levels in a cdc42 knockout is consistent with this idea.

We initiated these studies to characterize the role of the Cpcdc42 gene encoding a highly conserved Cdc42p-like protein. A high degree of structural conservation is common to Cdc42p-like proteins in eukaryotic organisms of distant relationship such as yeasts, flies, and mammals (Fig. 1). This was demonstrated in recent studies by heterologous complementation of S. cerevisiae cdc42 mutants with orthologs of W. dermatitidis (50) or A. gossypii (49) and also Caenorhabditis elegans (10), Drosophila melanogaster (35), and Homo sapiens (37). The structural similarities coincide with common functions within signal transduction pathways in all organisms investigated so far (20). However, these similarities (homology of interacting partners and influence on the cytoskeleton) do not reflect the differences in biological functions. Unlike in S. cerevisiae, S. pombe, and the closely related fungi C. albicans and A. gossypii, a deletion of Cdc42 is not lethal in more distantly related fungi such as W. dermatitidis, M. grisea, and P. marnieffii. The data presented in our studies support the idea that in contrast to yeast, Cdc42 is not essential in filamentous fungi. The multitude of processes for which Cdc42 of yeasts acts as a signal transducer seems to be divided in filamentous fungi to Cdc42 and a second Rho GTPase, Rac (14). So far, to our knowledge nothing is known about the signal pathways in which CDC42 is integrated and its downstream targets in filamentous fungi. Since the presence of CDC42 in C. purpurea has impact on, but is not essential for, vegetative growth and is indispensable for pathogenicity, we possess a valuable tool for the identification of downstream components of Cpcdc42 and especially of those involved in pathogenesis of C. purpurea.

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