In the filamentous phytopathogen Botrytis cinerea, the Ca\(^{2+}\)/calcineurin signaling cascade has been shown to play an important role in fungal growth, differentiation, and virulence. This study deals with the functional characterization of two components of this pathway, the putative calcium channel proteins Cch1 and Mid1. The cch1 and mid1 genes were deleted, and single and double knockout mutants were analyzed during different stages of the fungal life cycle. Our data indicate that Cch1 and Mid1 are functionally required for vegetative growth under conditions of low extracellular calcium, since the growth of both deletion mutants is strongly impaired when they are exposed to the Ca\(^{2+}\)-chelating agents EGTA and 1,2-bis(o-aminophenox)-ethane-N,N,N',N'\textsuperscript{-}tetraacetic acid (BAPTA). The impact of external Ca\(^{2+}\) was investigated by supplementing with CaCl\(_2\) and the ionophore A23187, both of which resulted in elevated growth for all mutants. However, deletion of either gene had no impact on germination, sporulation, hyphal morphology, or virulence. By use of the aequorin reporter system to measure intracellular calcium, a wide range of cellular processes, such as cell cycle progression, sporulation, spore germination, oriented hyphal tip growth, hyphal branching, gene expression, and circadian rhythms (4–7).

Intracellular calcium ions (Ca\(^{2+}\)) are important second messengers in all organisms. In resting states, the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\textsubscript{cyt}) is very low (about 50 to 100 nM). This calcium homeostasis is maintained mainly through calcium transporters and pump activity. Ca\(^{2+}\) signaling typically involves transient increases in intracellular Ca\(^{2+}\) concentrations originating from the extracellular medium or mobilization from internal compartments, such as the endoplasmic reticulum (ER), mitochondria, the Golgi apparatus, or the vacuole (1, 2). In response to external stresses, Ca\(^{2+}\) channels are opened, resulting in a rapid influx of Ca\(^{2+}\) ions (3). In yeasts and filamentous fungi, transient increases in [Ca\(^{2+}\)]\textsubscript{cyt} modulate signaling cascades and affect a wide range of cellular processes, such as cell cycle progression, sporulation, spore germination, oriented hyphal tip growth, hyphal branching, gene expression, and circadian rhythms (4–7). Changes in intracellular Ca\(^{2+}\) concentrations are sensed by the Ca\(^{2+}\) sensor protein calcmodulin (CaM), which binds cytosolic Ca\(^{2+}\) ions at EF-hand motifs and subsequently activates several Ca\(^{2+}\)/CaM-dependent enzymes, such as the phosphatase calcineurin (CN). Activation of CN results in dephosphorylation and nuclear translocation of the CN-responsive zinc finger transcription factor Crz1, which regulates the transcription of a set of Ca\(^{2+}\)/CN-dependent target genes via binding to CN-dependent responsive elements (4). Concomitantly, the [Ca\(^{2+}\)]\textsubscript{cyt} is reduced to the basal level (8).

The coordination and regulation of Ca\(^{2+}\) fluxes is essential for maintaining Ca\(^{2+}\) homeostasis. Efflux of Ca\(^{2+}\) from the cell can occur via ATPase-dependent Ca\(^{2+}\) pumps and antiporters (countertransporters), while Ca\(^{2+}\) entry occurs through transporters or channels (9–11). Ca\(^{2+}\)-permeable channels are responsible for the passive flow of Ca\(^{2+}\) across cell membranes into the cytosol. These channels are grouped into different families according to their mode of activation: mechanical, stretch-activated, and voltage-gated calcium channels (12).
mologs are quite similar in sequence and topological structure to human voltage-gated Ca\textsuperscript{2+} channels (19). All voltage-gated Ca\textsuperscript{2+}-permeable channels consist of four domains, each containing six transmembrane helices (S1 to S6), that tetramerize to form the core/aqueous pore of the Ca\textsuperscript{2+} channel (19). In addition, homologs of Cch1 exhibit a highly conserved acidic motif (glutamate residue) contributing to ionic selectivity (20). In yeast, Cch1 is localized in the plasma membrane, where it forms a complex with Mid1. Both proteins have been shown to be required for the uptake of extracellular Ca\textsuperscript{2+} in cells responding to mating pheromones (13, 21–24).

In filamentous fungi, deletion or silencing of Ca\textsuperscript{2+} channel-encoding genes revealed less-severe effects on growth and development than in yeast. In the rice blast fungus *Magnaporthe oryzae*, silencing of *mid1* and *cch1* resulted in only slight effects on growth rates, sporulation, and appressorium formation. The virulence of *mid1* mutants was not impaired, while *cch1* knockdown resulted in a slight reduction in virulence (25). In *Gibberella zeae*, Mid1 and Cch1 control mycelial growth and development and ascospore discharge, but *mid1* and *cch1* deletion mutants display a wild-type (WT)-like ability to infect wheat (26, 27). All these data show that the Ca\textsuperscript{2+}-permeable channels seem to be more involved in fungal development than in the interaction with host plants, with one main component: deletion of *mid1* in the phytopathogenic, biotrophic ascomycete *Claviceps purpurea* resulted in a complete loss of virulence (28), demonstrating the different roles Mid1 may play in some fungi. However, all *mid1* and *cch1* deletion mutants generated so far have one phenotype in common: the inability to grow under low-calcium (about 100 nM) conditions, indicating that they are part of the high-affinity calcium influx system (HACS) (29, 30). Besides HACS, there also exists a low-affinity calcium influx system (LACS), with the Ca\textsuperscript{2+} channel Fig1 as the main component. Fig1 becomes active in complete medium, when the Mid1-Cch1 HACS is proposed to be almost completely inhibited by CN (31).

In the necrotrophic plant pathogen *Botrytis cinerea*, several components of the Ca\textsuperscript{2+}/CN-dependent signaling cascade, such as the Go subunit BcG1 (*B. cinerea* Go1) of a heterotrimeric G protein (32–34), the phospholipase C (BcPlc1) (34), the protein phosphatase calcineurin (BcCN), and its intracellular regulator calcipressin (BcRcn1) (35), as well as the downstream transcription factor BcCrz1 (36), have been well characterized. Deletion of all components resulted in more or less severe defects in hyphal growth, fungal development, and gene regulation.

This report describes the molecular characterization of the putative stretch-activated Ca\textsuperscript{2+} channel Mid1 and the putative voltage-gated Ca\textsuperscript{2+} channel Cch1 in the phytopathogenic ascomycete *B. cinerea*. Single and double deletion mutants were analyzed for Ca\textsuperscript{2+} homeostasis, vegetative growth, and virulence. Localization studies on Mid1 were carried out to gain more insight into the function of this protein in this fungus.

**MATERIALS AND METHODS**

**Strains and culture conditions.** *B. cinerea* Pers.: Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] B05.10 is an isolate from *Vitis vinifera* (37) and was used as a host strain for transformation. All *B. cinerea* strains used in this study are listed in Table 1. Wild-type and mutant strains were grown on several complex media. Potato dextrose agar (Sigma-Aldrich) was supplemented with 10% homogenized leaves of French bean (*Phaseolus vulgaris*) (PDAB). Synthetic complete medium (CM) was made according to the method of Pontecorvo et al. (38). As minimal medium, modified Czapek-Dox (CD) medium (2% sucrose, 0.1% KH\textsubscript{2}PO\textsubscript{4}, 0.3% NaN\textsubscript{3}, 0.05% KCl, 0.05% MgSO\textsubscript{4}·7H\textsubscript{2}O, 0.002% FeSO\textsubscript{4}·7H\textsubscript{2}O [pH 5.0]) or GB5 medium (0.33% Gamborg's B5 medium [Duchefa Biochemical BV, Haarlem, The Netherlands], 2% glucose) was used. For condiation, the strains were incubated for 1 week at 20°C under light (12 h light/12 h darkness) conditions; for the formation of sclerotia, they were incubated for 2 weeks in continuous darkness. For DNA preparations, mycelium was grown for 3 days on CM agar with a cellulose acetate (Cellophane) overlay. Plate assays were performed using CM or CD agar with or without supplements [different concentrations of CaCl\textsubscript{2}, Congo red, calcofluor white, fluconazole, 1,2-bis(2-amino-phenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA; Sigma-Aldrich, St. Louis, MO), H\textsubscript{2}O\textsubscript{2}, EGTA (AppliChem GmbH, Darmstadt, Germany), and sodium dodecyl sulfate (SDS; MP Biomedicals Inc., Solon, OH)] as described by Schumacher et al. (36).

**Microscopic analyses.** Epifluorescence microscopy was carried out after incubation of germinating conidia for 16 to 24 h on glass slides in liquid GB5 medium supplemented with 2% glucose and 0.132 g/liter (NH\textsubscript{4})\textsubscript{2}HPO\textsubscript{4}. Fluorescence and light microscopy was performed with an Axios Imager.M2 microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). Different interference contrast (DIC) microscopy was used for bright-field images. Green fluorescent protein (GFP) fluorescence was examined with a freshly prepared 1:50 dilution of ER-Tracker Blue-White DPX (Molecular Probes, Inc.) in McIlvaine buffer (pH 7.2) (39) was added to germinated conidia prior to microscopy. For staining of the ER, 15 μl of a freshly prepared 1:50 dilution of ER-Tracker Blue-White DPX (Molecular Probes, Inc.) in McIlvaine buffer (pH 7.2) was added to the germinated conidia. Staining of nuclei or the ER was examined using filter set 49 DAPI shift free (excitation G 365, beam splitter FT 495, emission BP 445/50). For detection of nuclei, DNA was stained using the fluorescent dye Hoechst 33342 (20 μM; Thermo Scientific). For this purpose, 10 μl of freshly prepared Hoechst solution in McIlvaine buffer (pH 7.2) (39) was added to germinated conidia prior to microscopy. For staining of the ER, 15 μl of a freshly prepared 1:50 dilution of ER-Tracker Blue-White DPX (Molecular Probes, Inc.) in McIlvaine buffer (pH 7.2) was added to the germinated conidia. Staining of nuclei or the ER was examined using filter set 49 DAPI shift free (excitation G 365, beam splitter FT 495, emission BP 445/50). All images were captured with an AxiosCam MRm camera and were analyzed using the Axiosvision software package (release 4.5) (both from Carl Zeiss MicroImaging GmbH, Jena, Germany). Fluorescence was presented in green for GFP fluorescence and in blue or white for fluorescence using the 4',6-diamidino-2-phenylindole (DAPI) filter set.

**Standard molecular methods.** Fungal genomic DNA was isolated as described previously (40). For Southern blot analyses, the fungal DNA was transferred to Hybond N\textsuperscript{+} filters (Amersham Biosciences, Freiburg, Germany) after digestion with restriction enzymes and size separation on a 1% agarose gel according to the method of Sambrook et al. (41). Hybridization was carried out in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015

### Table 1: *Botrytis cinerea* strains used in this study

| B. cinerea strain | Characteristics | Source or reference |
|-------------------|-----------------|---------------------|
| WT:B05.10         | Isolate from *Vitis vinifera* (Germany); MAT1-1 | 37 |
| Δmid1             | B05.10 Δmid1::hph; homokaryon | This study |
| Δcch1             | B05.10 Δcch1::nat; homokaryon | This study |
| Δcch1 Δmid1       | B05.10 Δcch1::nat Δmid1::hph; homokaryon | This study |
| Δmid1::mid1::gfp   | B05.10 Δmid1::hph Δmid1::gfp::nat; heterokaryon | This study |
| Δmid1::gfp-mid1    | B05.10 Δmid1::hph gfp-mid1::nat; heterokaryon | This study |
| WT::aeq           | B05.10 aeqS::hph; heterokaryon | This study |
| Δmid1::aeq        | B05.10 Δmid1::hph aeqS::nat; heterokaryon | This study |
| Δcch1::aeq        | B05.10 Δcch1::nat aeqS::hph; heterokaryon | This study |

**TABLE 1** *Botrytis cinerea* strains used in this study

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M sodium citrate), 5 × Denhardt’s solution, 0.1% SDS, and 50 mM phosphate buffer (pH 6.6) at 65°C in the presence of a random-primed [α-32P]dCTP-labeled probe. The membranes were washed once before being exposed to autoradiographic film. For sequence analyses, LaserGene software (version 10; DNASTar, Madison, WI) was used. To provide further evidence for N-glycosylation sites (http://www.expasy.org/protomise) and transmembrane domain prediction (http://www.sbc.su.se/~miklos/DAS/), different software was used. All primers used in this study are listed in Table S1 in the supplemental material.

Vector cloning. For construction of the pΔch1 vector, plasmid pNR1 (42), carrying the Streptomyces noursei nat1 gene encoding nourseothricin acetyltransferase under the control of the Aspergillus nidulans oliC promoter, was used as a basal vector. The gene fragments were amplified by PCR with primers derived from the genomic sequence of B. cinerea B05.10 (B0510_6305) and containing artificial restriction sites for further cloning. A 1.1-kb PCR fragment was amplified from the cch1 5′ region using primers 1 and 2, and a 0.9-kb fragment of the 3′ untranslated region was generated as a second flank using primers 3 and 4 (Fig. 1A). Both PCR products were cloned into pCR2.1-TOPO (Invitrogen, San Diego, CA) or 70 μg/ml of hygromycin B (Invitrogen, San Diego, CA) or 70 μg/ml of nourseothricin (Werner Bio-Agens, Jena, Germany). Single conidial isolates were obtained by spread- ing the T2 strain were transformed with the hygR-containing fragment, and the Δmid1 T2 mutant strain was transformed with the natR-containing fragment, resulting in the aeq-, Δch1 aeq, and Δmid1 aeq strains, respectively. For each transformation approach, at least two independent primary transformants were generated. Homologous integration at the ben11a locus was confirmed. Furthermore, the gpd promoter of vector pAEQS1-15 was replaced by the constitutively active oliC promoter, since the former was not functional in B. cinerea. As described above, yeast DNA was isolated and retransformed into E. coli. Plasmid DNA from single colonies was sequenced, resulting in the final vector pNAH-OQT (oliC-prom::aegS::trpC-term). By using this vector as a basal vector, a natR resistance cassette was integrated by cotransformation of yeast with pNAH-OQT (SphI/Smal) and a PCR amplification product of primers 29 and 30, creating pNAH-OQT. Both plasmids were linearized with SacII/Apal and transformed into B. cinerea strains. The wild-type strain B05.10 and the Δch1 T2 strain were transformed with the hygR-containing fragment, and the Δmid1 T1 mutant strain was transformed with the natR-containing fragment, resulting in the aeq-, Δch1 aeq, and Δmid1 aeq strains, respectively. For each transformation approach, at least two independent primary transformants were generated. Homologous integration at the ben11a locus was shown by diagnostic PCR using primers 31 and 32 for the 5′ flank and primers 33 and 34 or 35 for the 3′ flank.

Transformation of B. cinerea. For transformation, strains were grown overnight in liquid malt extract medium (0.5% glucose, 1.5% malt extract [Difco], 0.1% cassein peptone, 0.1% Casamino Acids, 0.1% yeast extract, 0.02% Na-nucleinate) or on CM with overlying Cellophane. Protoplasts were generated using a mixture of Glucanex 200G (Novozymes, Denmark), lysing enzyme (Sigma-Aldrich, St. Louis, MO), and Yatalase (TaKaRa Bio Inc., Shiga, Japan) and were transformed according to the work of Siewers et al. (49). Resistant colonies were transferred to plates containing CM agar complemented with 70 μg/ml of hygromycin B (Invitrogen, San Diego, CA) or 70 μg/ml of nourseothricin (Werner Bio-Agens, Jena, Germany). Single conidial isolates were obtained by spreading conidial suspensions on GB5 plates containing 70 μg/ml of hygromycin B or nourseothricin. Single germinated conidia were transferred individually to new plates containing the selection marker for the generation of homokaryotic transformants.

Cytosolic Ca2+ measurements. For [Ca2+]1,4 measurements, aeqS-expressing B. cinerea strains were grown in different culture media. Spore suspensions were used for inoculation of a 300-ml flask containing 100 ml GB5 medium supplemented with 2% glucose and 0.132 g/liter (NH4)2HPO4 and cultures were grown overnight at 170 rpm and 18 to 20°C. Cultures were harvested by centrifugation. Five milliliters of fresh medium was applied, and 2 ml of the culture was incubated on a shaker with the cofactor coelenterazine (coelenterazine c; Sigma-Aldrich Chemie, Steinheim, Germany) at 2.5 μM. Coelenterazine was dissolved in

Homologous recombination system in yeast (43). For construction of the gfp-mid1 fragment, the coding region of mid1 was amplified using primers 23 and 24, which contain overlapping sequences homologous to the glucanase terminator and gfp (encoding codon-optimized GFP) (46), respectively, of the pNAH-OGG vector (47). This vector contains flanks mediating the replacement of the gene encoding the nitrite reductase and therefore ensuring integration at a known locus. The PCR product and the NotI-digested plasmid pNAH-OOG were cotransformed into S. cerevisiae strain FY834. Plasmid DNA from single colonies was isolated, transformed into Escherichia coli, and sequenced, resulting in the final vector pNAH-OmgOG. Plasmid pNAH-OmidGG, containing the same components but with the mid1 gene cloned upstream of gfp, was constructed in a similar way by using primers 25 and 26 for PCR amplification and the pNAH-OOG vector restricted with NcoI for yeast transformation. After linearization of both vectors with SacII/Apal, the resulting fragments, containing the ben11a flanks, natR, and the gfp fusion constructs flanked by the oliC promoter and the glucanase terminator, were transformed into the WT strain B05.10 and the Δmid1 T1 strain. Integration of the transformation constructs was analyzed by diagnostic PCR (data not shown).

To use aequorin as an intracellular Ca2+ sensor, the aeq and the trpC terminator of vector pAEQS1-15 (48) were amplified using primers 27 and 28. The resulting fragment was cotransformed into yeast strain FY834 with plasmid pNAH-OGG (47), which was restricted with NotI/Spel in advance. By this procedure, homologous integration at the ben11a locus was ensured. Furthermore, the gpd promoter of vector pAEQS1-15 was replaced by the constitutively active oliC promoter, since the former was not functional in B. cinerea. As described above, yeast DNA was isolated and retransformed into E. coli. Plasmid DNA from single colonies was sequenced, resulting in the final vector pNAH-OQT (oliC-prom::aegS::trpC-term). By using this vector as a basal vector, a natR resistance cassette was integrated by cotransformation of yeast with pNAH-OQT (SphI/Smal) and a PCR amplification product of primers 29 and 30, creating pNAH-OQT. Both plasmids were linearized with SacII/Apal and transformed into B. cinerea strains. The wild-type strain B05.10 and the Δch1 T2 strain were transformed with the hygR-containing fragment, and the Δmid1 T1 mutant strain was transformed with the natR-containing fragment, resulting in the aeq-, Δch1 aeq, and Δmid1 aeq strains, respectively. For each transformation approach, at least two independent primary transformants were generated. Homologous integration at the ben11a locus was shown by diagnostic PCR using primers 31 and 32 for the 5′ flank and primers 33 and 34 or 35 for the 3′ flank.

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FIG 1 Gene replacement in the *B. cinerea* Δmid1, Δcch1, and Δcch1 Δmid1 strains. (A) Deletion strategies for the Δcch1 (top) and Δmid1 (bottom) strains. All primers used for cloning of the replacement vectors and for diagnostic PCR analyses to prove homologous integration are indicated by numbers from 1 to 20, and their use is described further in Materials and Methods. Introns are depicted as open vertical bars in arrows representing the genes. Restriction sites for cloning and for Southern blot analyses are indicated, as are expected fragment sizes and probes for the hybridization. (Top) Physical maps of wild-type (WT; strain B05.10) cch1 and the Δcch1 locus. The wild-type *B. cinerea* strain B05.10 was transformed with the cch1 replacement fragment (derived from cloning of both flanking regions into vector pNR1 containing the *nat*<sup>R</sup> resistance cassette), resulting in the Δcch1 strain via homologous recombination and insertion of *nat*<sup>R</sup>. (Bottom) Physical maps of the WT mid1 locus and the mutated Δmid1 locus. The mid1 gene was replaced by the hph<sup>R</sup> cassette (derived from vector pOlIHP) in the opposite direction via homologous recombination. (B) Southern blot analyses of the Δmid1, Δcch1, and Δcch1 Δmid1 mutants. Two to four different mutants were tested for any additional ectopic integration of the respective resistance cassettes. The wild type, the Δmid1 T1 and T7 strains, all four Δcch1 mutants, and the Δcch1 Δmid1 T3 and T7 strains each displayed just one hybridizing fragment with the expected size (for comparison, see panel A). Asterisks indicate strains which were used for further phenotypic analyses.
methanol before use. After 1 h, preincubation was stopped by centrifugation and the addition of 2 ml fresh medium. A flat-bottom 96-well plate was inoculated with 100 μl of the young mycelium per well. Aequorin luminescence was detected using a Berthold Technologies TriStar plate luminometer, driver version 1.05, equipped with three injectors, each dispensing 100 μl of liquid at defined time points. The light output was recorded in relative luminescence units (RLU). Amiodarone (AMD) (amiodarone hydrochloride; Sigma-Aldrich Chemie, Steinheim, Germany) was dissolved in 12% ethanol (EtOH) according to the method of Courchesne and Ozturk (50). GB5 medium containing 12% EtOH was used as a control for AMD treatment. For calculation of the cytosolic Ca²⁺-concentration at the end of each measurement, the total luminescence was measured for 120 s after complete discharge of the present aequorin by injection of 100 μl 4 M CaCl₂ in 10% EtOH (48, 51). The RLU values were multiplied by 1.24 to correct for quenching by ethanol. RLU values were then converted to [Ca²⁺]ₜₜ by using the following empirically derived equations: pCa = 0.332588 × (−log₁₀ K) + 5.5593, and K = luminescence (in RLU) s⁻¹/total luminescence (in RLU) (48, 51).

RESULTS

Identification of *B. cinerea* Mid1 and Cch1 homologs. In the *B. cinerea* genome sequence (Broad Institute), we found one gene (B0510_6305) with significant homology to the yeast *cch1* gene. The predicted amino acid sequence (2,161 amino acids) contains all four hydrophobic transmembrane domains (Fig. 2A), including the highly conserved acidic motifs (N, E, E, E) required for calcium binding, and four putative voltage-sensing regions.

Furthermore, we identified one gene (B0510_470) with high homology to the yeast *mid1* gene. The corresponding gene product codes for a protein with 675 amino acids. Several conserved domains, such as four putative TM regions, an EF-hand-like structure, 16 N-glycosylation sites, and 14 cysteine residues, located mostly in the C-terminal region, were found in the protein sequence. Additionally, a putative N-terminal signal peptide was found in the *mid1* gene product (Fig. 2B). All domains are predicted to be essential for the activity and localization of Mid1 in *S. cerevisiae* (13, 16).

Knockout of *mid1* and *cch1* had no impact on conidiation, germination, or virulence. The *B. cinerea* homologs of both *mid1* and *cch1* were cloned, their intron/exon structures were confirmed, and single and double deletion mutants were generated (Fig. 1A). After single-spore isolation, homokaryotic mutants no longer contained the respective wild-type allele or any ectopic integrations of the resistance cassette, as shown by diagnostic PCR and Southern blot analyses (Fig. 1B). For each deletion (Δ*mid1*, Δ*cch1*, Δ*cch1 Δmid1*), one of the phenotypically identical mutants was used for further characterization.

Neither spore germination under sugar-inducing conditions (GB5 medium) or on hydrophobic surfaces (polypropylene foil) nor conidial anastomosis tube (CAT) formation and hyphal fusion were affected in any of the three mutants. The hyphal morphology of young germ tubes as well as older hyphae was the same as for the wild type. Furthermore, the virulence of the deletion mutants was not impaired: the single and double knockout mutants were able to penetrate (tested in an onion epidermis penetration assay) and to infect living bean plants without any noticeable differences from the wild type. Primary and spreading lesions were formed, and *in planta* sporulation occurred during a 1-week period in a wild-type-like manner (data not shown).

The growth defect in axenic culture was restored by supplementation with external calcium. However, the growth of the *mid1*, *cch1*, and *cch1 mid1* deletion mutants was significantly affected in axenic culture. On complete medium (CM), all three strains were similarly retarded in growth (Fig. 3A), achieving growth rates of merely 50 to 60% of the wild-type growth rate per day (Fig. 3B). Sector formation in the mutant colonies was often observed after several days, when the wild type had already reached the edge of the agar plate. Diagnostic PCR proved that the mycelium in these sectors was still homokaryotic and did not contain wild-type *mid1* or *cch1* alleles. Inoculation of new agar plates with mycelial plugs derived from faster-growing sectors resulted in the same sector-forming growth pattern. Conidiogenesis (un-
der light conditions) and the formation of sclerotia (during continuous darkness) were unaffected in the mutants (Fig. 3A). Despite the lower growth rate on complete medium, the mutants showed wild-type-like responses to stressors such as SDS (inducing membrane stress), Congo red, calcofluor white (both inducing cell wall stress), H2O2 (inducing oxidative stress), and fluconazole (an antifungal drug) used as CM supplements (data not shown).

On minimal medium (CD medium), the phenotype of the mutants was even more severe. The growth rates of single and double mutants were significantly affected (30 to 40% of the WT growth rate) (Fig. 3B). However, sector formation, differences in hyphal density, and penetration into the agar medium render assessment of the observed growth defects difficult. Therefore, biomass production by all strains was also compared in liquid cultures. The mutants generated just about half of the biomass of the wild type, confirming the results of our measurement of colony diameters in plate assays (Fig. 3C). The phenotypic growth retardation was partially reversed by the addition of low concentrations of external calcium (50 to 100 mM CaCl2) to the B. cinerea cch1, mid1, and cch1 mid1 deletion mutants, indicating that these putative channels are indeed involved in calcium transport (Fig. 3 and 4). However, higher concentrations of CaCl2 caused significant decreases in the diameters of both wild-type and mutant colonies.

To study the effect of external Ca2+ limitation, all strains were grown on minimal medium in the presence or absence of small amounts of the Ca2+/H+ chelating agent EGTA. The wild type was only slightly affected when exposed to 10 mM EGTA, while all mutants showed drastically reduced growth rates and were able to grow only about 2 mm in 1 week. A 15 mM concentration of EGTA caused complete failure of colony extension (Fig. 4). A similar result was observed with 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), a more calcium specific chelating agent: addition of 1 mM BAPTA to minimal medium fully abolished the growth of all mutants, while the wild type was able to grow about 4 cm in colony diameter in 5 days (Fig. 5). Taken together, deletion of both Mid1 and Cch1 resulted in significantly reduced growth under Ca2+/H+ limiting conditions, suggesting that these mutants are not able to compensate for the extracellular limitation by mobilizing internally stored Ca2+.

In Aspergillus awamori, the calcium ionophore A23187 was shown to increase intracellular Ca2+ levels by acting as a mobile ion carrier, as demonstrated by increased aequorin luminescence (48). To study the effect of this ionophore in B. cinerea, the growth of the wild type and all mutants was compared on a medium amended with A23187. Filter discs with different concentrations of the calcium ionophore, or with the solvent EtOH
as a control, were placed on minimal medium (CD) agar plates before the plates were inoculated with mycelium plugs (Fig. 6). The growth of all strains was inhibited in a circle with a width of about 2 mm around the ionophore-containing filter discs, probably due to toxic effects of the ionophore itself. The wild type grew unhindered over the EtOH control discs and expanded in more or less circular growth over the entire plates, while the ionophore-containing discs caused a zone of growth inhibition. In contrast, the mutant strains were restricted in growth directed to the control discs but displayed improved growth near the filter discs containing the calcium ionophore. The effect of calcium starvation near the EtOH control discs was reversed by addition of 50 mM CaCl₂ to the medium. In this case, all three mutants displayed growth patterns similar to that of the wild type, indicating that the increase in growth toward the ionophore was indeed due to calcium availability. Further increases in the external calcium concentration up to 400 mM did not change the results shown in Fig. 4.

To summarize, both Mid1 and Cch1 have an impact on vegetative growth under calcium-limiting conditions. The growth rates of the deletion mutants were significantly improved when the \([\text{Ca}^{2+}]_{\text{cyt}}\) levels were artificially increased by the addition of external CaCl₂ or the calcium ionophore A23187.

**Mid1 is localized in net-like filaments and around the nuclei.** So far, the subcellular localization of Mid1 has not been shown in any filamentous fungus. In yeast, Mid1 is located in the plasma

**FIG 4** Depiction of the calcium rescue phenotype of *B. cinerea* Δmid1, Δcch1, and Δcch1 Δmid1 mutants in comparison to the wild-type strain B05.10. Strains were grown on minimal medium (CD) alone or containing either the indicated amounts of CaCl₂ at increasing concentrations (from 50 to 400 mM) or the Ca²⁺-chelating agent EGTA (10 or 15 mM). (A) Graphic presentation of relative growth 2 days after inoculation. Strain were incubated under light/dark conditions (12 h/12 h). The growth of the wild-type strain B05.10 on a control medium (CD) was set at 100%; that for all other strains and conditions was calculated proportionally. Mean values for three biological replicates with at least four plates per strain and condition were used for the calculation of percentages. Error bars, standard deviations. High standard deviations result from enhanced sector formation on minimal medium. (B) Representative plates after 3 days postinoculation.

**FIG 5** Effect of the calcium chelator BAPTA (1 mM) on growth on minimal medium (CD). The indicated strains were inoculated with mycelium plugs and were grown for 3 days under day/night conditions.
membrane as well as in the membrane of the endoplasmic reticulum (ER) (16). To show where Mid1 localizes in B. cinerea, mid1 was fused to the N terminus of codon-optimized gfp, and the fusion protein was expressed in two genetic backgrounds, the wild-type strain B05.10 and the mid1 deletion mutant. The fusion construct was functional, as evidenced by the fact that it restored wild-type-like growth rates in the mid1 background (Fig. 3Band 5). The GFP signal in both strains (the WT mid1-gfp strain and the mid1 deletion mutant) was most prominent around the nuclei in net-like filaments, which were distributed over the whole hyphae of young germinated conidia (Fig. 7A). Fluorescence signals for the fusion protein were never observed in the plasma membranes of germinated conidia and protoplasts. To scrutinize this aspect, we also cloned mid1 to the C terminus of gfp and likewise expressed the fusion construct in both backgrounds. The distribution of fluorescence signals was similar to that described above. Again network-like filaments and clearances in the nuclei were observed over the full length of the hyphae examined (Fig. 7B). However, the fusion of mid1 to the C terminus of gfp did not result in complementation of the mid1 deletion phenotype under calcium depletion conditions. To further examine our hypothesis that the network-like filaments probably represent the ER and the nuclear envelope, germinated hyphae were stained with ER-Tracker Blue-White DPX (Fig. 7C). The fluorescent signals of the

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**FIG 6** Effect of increasing the cytosolic Ca**2+** concentration by the presence of the calcium ionophore A23187 on the growth of B. cinerea Δmid1, Δcch1, and Δcch1 Δmid1 mutants in comparison to that of the wild-type strain B05.10. The indicated mutant strains and the wild-type strain B05.10 were grown on minimal medium (CD) with or without additional CaCl2 (50 mM) for 4 days. Circular filter discs supplied with a 10-µl droplet of EtOH (control) or with the calcium ionophore A23187 at 4.725 or 9.5 mM were laid on the agar plates just before inoculation. The extent of growth is outlined by white dots. Schematic views of growth rates in the indicated directions are shown below the plates. All strains exhibited similar zones of inhibition around the ionophore-containing filter discs. Apart from that, the wild type showed circular growth in all directions on CD medium, whereas the mutant strains displayed less growth in the direction of the control than in the direction of the ionophore filter discs. The addition of 50 mM CaCl2 reversed the effect. The mutants’ defect in growth toward the control was rescued, and like the wild type, they displayed approximately circular growth, except for the indicated inhibition zones of all strains around the ionophore-containing discs.
ER-Tracker colocalized with Mid1-GFP. In comparison, the non-transformed wild type itself displayed no fluorescence with the same microscopic settings (see Fig. S1 in the supplemental material). Additionally, germinated hyphae were stained with Hoechst 33342, a dye that is able to bind to DNA and to stain most prominently the nuclei (see Fig. S1). The GFP signal was not detectable in the nuclei but in their immediate vicinity.

To summarize, Mid1 in B. cinerea is localized in net-like filaments, probably the ER and the nuclear envelope, but was not seen in the plasma membrane under the conditions used.

Cytosolic calcium measurement using aequorin. To further investigate the role both putative Ca\(^{2+}\) channels play in regulating the intracellular calcium level of B. cinerea, the codon-optimized aequorin gene, encoding the Ca\(^{2+}\) reporter aequorin (48), was introduced into the wild type and the single knockout mutants. By using the aequorin reporter gene, changes in the [Ca\(^{2+}\)]\(_{cyt}\) in response to extracellular stress should be detected. The aequorin reporter introduced was shown to be functional, since high peaks of relative luminescence units (RLU) have been observed in response to extracellular stressors. These were used for calculation of the [Ca\(^{2+}\)]\(_{cyt}\). The resting cytosolic calcium concentration of B. cinerea strains in GB5 medium was about 50 nM (Fig. 8). In yeast, it has been shown that amiodarone (AMD) affects calcium homeostasis and causes a dramatic increase in the cytoplasmic calcium concentration, leading to cell death after short exposures (50). The rise in [Ca\(^{2+}\)]\(_{cyt}\) after AMD treatment appeared to result from both release of Ca\(^{2+}\) from intracellular stores and stimulation of caffeine-sensitive calcium entry. S. cerevisiae Δmid1 mutants showed a lower response to AMD than the wild type or Δech1 mutants, suggesting Mid1 activity in the AMD response.

We performed similar experiments and analyzed the aequorin luminescence of wild-type B. cinerea and both single mutants expressing the reporter gene in response to AMD treatment (Fig. 8). Quantitative monitoring of [Ca\(^{2+}\)]\(_{cyt}\) revealed a dramatic increase up to 1 μM after AMD treatment, whereas just a small increase to 0.2 μM was observed after addition of a control medium (mechanical perturbation through injection). The increase in [Ca\(^{2+}\)]\(_{cyt}\) occurred within seconds after injection. Increasing AMD concentrations resulted in increased aequorin signals for the WT aeq strain (data not shown). Unexpectedly, no differences could be observed between the WT aeq strain, the Δbcmid1 aeq strain, and the Δbcch1 aeq strain (Fig. 8). A variety of growth conditions were tested, since the deletion mutants grew differently on complete and minimal media (data not shown). We observed differences in the basal intracellular calcium levels of all strains depending on the culture medium before aequorin measurement. However, for both deletion mutants, the resting [Ca\(^{2+}\)]\(_{cyt}\) was similar to that of the wild type, although the mutants revealed reduced growth, indicating conditions of intracellular calcium starvation.

**DISCUSSION**

In B. cinerea, several components of the Ca\(^{2+}\) signaling pathway have been identified and characterized (34–36). It has been shown that the Ca\(^{2+}\)/calcineurin (CN) pathway is involved mainly in processes such as hyphal growth and septation and, to a lesser extent, in differentiation processes such as spore production, germination, and sexual development. Recent studies on functional characterization of the phospholipase C (34), CN, the CN regulator Rcn1 (35), and the transcription factor Crz1 (36) in B. cinerea indicate that some components of the calcium signaling cascade also play a role in virulence: gene deletions resulted either in reduced virulence or in completely avirulent strains.

However, the role of Ca\(^{2+}\) channels for the maintenance of intracellular Ca\(^{2+}\) homeostasis, on the one hand, and the increases in Ca\(^{2+}\) levels upon activation of the CN-dependent signaling pathway, on the other hand, are still not well understood. To maintain high Ca\(^{2+}\) levels in subcellular storage organelles, such as vacuoles and the ER, several Ca\(^{2+}\) pumps, transporters, and channels in different membranes must be involved. The presence of those channel proteins in the plasma membrane enables growing hyphae to load the intracellular stores from external sources.

**FIG 7** Localization studies of the Mid1 protein in B. cinerea. (A) The Δmid1 strain was transformed with a mid1-gfp construct, where mid1 was fused N-terminally to gfp and was expressed under the control of the constitutively active oliC promoter. In germinated conidia (germination occurred for 20 h in GB5 medium in darkness), fluorescence was observed in network-like filaments and around the nuclei. Staining of nuclei by Hoechst 33342 is depicted in Fig. S1 in the supplemental material. (B) The Δmid1 strain was transformed with a gfp-mid1 construct, where mid1 was fused C-terminally to gfp and was expressed under the control of the constitutively active oliC promoter. The fluorescence signal observed was similar to that in panel A. (C) Staining of the Δmid1/mid1-gfp strain with ER-Tracker. In the top three images, the intracellular GFP-fluorescent structures are mostly consistent with the signal of the ER marker (white), as shown in the overlay (merge; false color blue for ER-Tracker). At the bottom, a DIC image of the germinated conidia is shown. Scale bars, 10 μm.
Genes involved in Ca\(^{2+}\) influx and efflux have been identified in many yeasts and filamentous fungi (19, 52). Recently, the roles of the two calcium channels, Mid1 and Cch1, have been studied in diverse fungi. The presence of single genes encoding Cch1 and Mid1 homologs in most fungal genomes suggests a conserved function for Cch1/Mid1 Ca\(^{2+}\) channels. The results presented here demonstrate that deletion of the corresponding genes in B. cinerea affects vegetative growth under calcium-limiting conditions (Fig. 3 to 6). The deletion mutants were unable to grow at low extracellular Ca\(^{2+}\) concentrations, as has also been shown for other fungi. Addition of the calcium-chelating agents EGTA and BAPTA, which binds calcium even more specifically than EGTA, resulted in strong growth defects for the B. cinerea channel mutants, indicating a role of these proteins in providing the cell with sufficient Ca\(^{2+}\) for normal growth in low-calcium environments. Hypersensitivity to low exogenous Ca\(^{2+}\) levels is consistent with an inability to regulate a homeostatic level transduced through the Ca\(^{2+}\)/CN signaling system. We propose that Mid1 and Cch1 play a role in Ca\(^{2+}\) homeostasis, because mid1 and cch1 deletion mutants were unable to respond to lower Ca\(^{2+}\) levels. However, the restoration of growth at high calcium concentrations indicates that Ca\(^{2+}\) ions can enter the plasma membrane and elevate intracellular Ca\(^{2+}\) levels to allow wild-type-like growth by the activation of another channel (e.g., Fig1).

Differences between B. cinerea, on the one hand, and N. crassa, C. purpurea, and G. zeae, on the other hand, were seen upon treatment with the ionophore A23187, which was shown to act as a mobile ion carrier (27, 28, 53). In the latter three fungi, addition of the calcium ionophore A23187 and subsequent increases in the ionophore (Fig. 6), suggesting that the increased [Ca\(^{2+}\)]\(_{\text{cyt}}\) levels are sufficient to activate downstream signaling cascades and to promote wild-type-like hyphal growth. Therefore, B. cinerea mid1 and cch1 deletion mutants were the only fungal channel mutants so far discovered whose growth rate was improved by addition of the ionophore A23187.

In contrast, addition of external CaCl\(_2\) resulted in increased growth rates for the B. cinerea mutants, similar to the rates described for other fungi. For example, in yeast, the mating-induced death phenotype is rescued by elevated Ca\(^{2+}\) (13, 21).

A characteristic feature of single and double mid1 and cch1 mutants was sector formation when strains were grown for several days on agar plates. Since the mutants displayed wild-type-like growth on minimal medium with small amounts of CaCl\(_2\), we suggested that sector formation is due to calcium starvation and hyphal polarity effects, as described for mid1 or cch1 deletion mutants in other filamentous fungi (26, 27, 53). However, when the cell wall was stained with calcofluor white, no differences from the wild type in hyphal morphology were observed, indicating that there were no defects in hyphal polarity or septation. Defects in the vegetative growth of B. cinerea mutants may also be due to alterations of cell wall composition. Assays for susceptibility to calcofluor white and Congo red, both of which interfere with the construction of the cell wall (54), exhibited no significant effect on the growth rates of mid1 and cch1 deletion mutants, suggesting that these channels do not play an important role in the cell wall integrity pathway in B. cinerea. In contrast, mid1 deletion mutants of A. nidulans were more resistant to these compounds (55), while C. purpurea mutants were more sensitive and showed impaired growth (28).

Virulence was unaffected in the B. cinerea single and double deletion mutants. The cch1 knockdown mutants of M. oryzae, which show a drastic reduction in the formation of conidia and appressoria, were still capable of infection, indicating that the reduced numbers of appressoria formed were still functional (25). In contrast, knockdown of mid1 in these experiments resulted in only a slight change of phenotype, and the mutants were fully pathogenic, a result that may indicate an uncoupled function of these channels. In G. zeae, the channel mutants showed slightly slower progression of disease. However, Cavinder and coworkers suggest that reduced conidial production and reduced ascospore discharge are the reasons for limited lesion spreading by these mutants (26, 27). Only in C. purpurea does Mid1 play a major role in the plant-fungus interaction, since mid1 deletion mutants were apathogenic (28). These results demonstrate the divergence of Mid1 and Cch1 function between different filamentous fungi. In B. cinerea, these channels do not play an essential role in any stage of the plant infection process.

Mid1 localization studies revealed exclusive localization in network-like filaments (Fig. 7), in a manner similar to that described for the ER in other filamentous fungi (36). The visualization of the GFP signal in internal membranes for both the N-terminal and the...
C-terminal fusions of GFP to Mld1 underlines the possibility that Mld1 in *B. cinerea* is an intracellular calcium channel, probably located in the ER, as shown by colocalization with an ER marker. Fluorescence at the nuclear envelope was demonstrated by costaining with Hoechst 33342. No fluorescence was observed at the plasma membrane under any condition tested. This is the first report of subcellular localization of Mld1 in a filamentous fungus.

Although we showed localization of Mld1 in the ER, this protein possesses no ER retention signals. Integral predictions of protein localizations by different computer programs (ProtComp, version 9.0; WoLF PSORT) revealed a preference for plasma membrane localization. In yeast, Mld1 seems to be located in the ER and the plasma membrane (15). However, the Mld1-GFP fusion protein displayed just a patchy distribution in the yeast plasma membrane, in contrast to continuous distribution of the plasma membrane marker protein Pma1. The patchy distribution of the fluorescent signal in yeast was explained by a probable association of Mld1 with some intracellular and/or extracellular matrix components or integral membrane proteins (15). Since Cch1 was suggested to interact physically with Mld1, we tried to clone a similar *cch1-gfp* construct in order to study the subcellular localization of Cch1 in *B. cinerea*. However, reamplification of the vector in *E. coli* never resulted in positive clones, as shown for the *S. cerevisiae* and *Cryptococcus neoformans cch1* genes (22, 57).

In yeast, interaction between Cch1 and Mld1 has been shown by coimmunoprecipitation. However, these proteins can still respond to some stimuli differently, suggesting common but also individual functions of the two channels (24, 50). Recently, it has been shown by a yeast two-hybrid approach that these proteins also interact in *A. nidulans* (55). In *G. zeae*, the double mutant of both channel proteins exhibited a stronger phenotype than the single *mid1* deletion mutant (26), supporting the idea of both dependent and independent roles for Mld1 and Cch1. In *B. cinerea*, the single and double knockout mutants displayed similar phenotypes under all conditions tested, suggesting that these two proteins act in a complex and have common functions, as also reported for *mid1* and *cch1* knockout mutants in *Candida albicans* and *S. cerevisiae* (21–23, 58). To study a putative interaction between these two proteins in *B. cinerea*, we used a split ubiquitin-based yeast two-hybrid system (Dualsystems). However, in contrast to the results for *A. nidulans*, we could not provide any evidence for an interaction of the two channel proteins by this method (data not shown).

To be able to quantify intracellular Ca$^{2+}$ concentrations, we established the aequorin reporter system for *B. cinerea*. The functionality of the calcium detection system has been demonstrated, and future studies will focus on the different behaviors of mutants defective in different calcium cascade components under various stress conditions, such as Ca$^{2+}$, pH, and oxidative stresses, or stresses induced by using calcium channel blockers (nifedipine, diltiazem), or the calcium ionophore A23187. However, we were unable to present data confirming the hypothesis of separate functions of Mld1 and Cch1 by AMD treatment, as shown by Courchesne and Ozturk for *S. cerevisiae* (50). Another study showed enhanced growth sensitivity of Δ*mld1* and Δ*cch1* mutants to AMD over that of the wild type, also revealing discrepancies between the effects of this drug on Mld1 and Cch1 protein functions (59). Recently, researchers also studied the influence of AMD on calcium and pH homeostasis in *Aspergillus niger* by using aequorin as a reporter (60). They showed that a characteristic calcium signature and elevated cytosolic calcium concentrations are accompanied by intracellular acidification after AMD treatment and that deletion of the calcium P-type ATPases, PmRA and PmCA, resulted in mutants hypersensitive to AMD.

Recently, a new component of the calcium influx system, Fig1, has been characterized. This channel is suggested to be active in the absence/inhibition of Cch1 and Mld1 activity (31, 61, 62). In contrast to Cch1 and Mld1, Fig1 seems to be important for the low-affinity calcium influx system (LACS). A homologous gene (*B0510_2417*) has been identified in the genome of *B. cinerea*. It is possible that the encoded protein acts as the major calcium supplier for the maintenance of intracellular homeostasis in *B. cinerea* under the conditions described here.

The results presented here, in addition to those from our previous studies, show the functional flexibility of components of the calcium signaling machinery among different fungi. Even within the phytopathogenic ascomycetes, the roles of HACS channels have diverged. In *B. cinerea*, all three mutants always displayed similar phenotypes, suggesting that the two gene products interact in the same pathway. Apparently, Mld1 is localized in an intracellular membrane system, probably the ER. However, a still open question is whether Mld1 function is completely restricted to the ER, since the observed phenotype also indicates an association with calcium influx via the plasma membrane. Future studies will focus on the role Fig1 plays in the calcium pathway of *B. cinerea*.

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