Functional Characterization of CgCTR2, a Putative Vacuole Copper Transporter That Is Involved in Germination and Pathogenicity in *Colletotrichum gloeosporioides*†

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Copper is a cofactor and transition metal involved in redox reactions that are essential in all eukaryotes. Here, we report that a vacuolar copper transporter that is highly expressed in resting spores is involved in germination and pathogenicity in the plant pathogen *Colletotrichum gloeosporioides*. A screen of *C. gloeosporioides* transformants obtained by means of a promoterless green fluorescent protein (GFP) construct led to the identification of transformant N159 in which GFP signal was observed in spores. The transforming vector was inserted 70 bp upstream of a putative gene with homology to the *Saccharomyces cerevisiae* vacuolar copper transporter gene *CTR2*. The *C. gloeosporioides CTR2* (*CgCTR2*) gene fully complemented growth defects of yeast *ctr2Δ* mutants, and a *CgCTR2*-cyan fluorescent protein (CFP) fusion protein accumulated in vacuole membranes, confirming the function of the protein as a vacuolar copper transporter. Expression analysis indicated that *CgCTR2* transcript is abundant in resting conidia and during germination in rich medium and down-regulated during “pathogenic” germination and the early stages of plant infection. *CgCTR2* overexpression and silencing mutants were generated and characterized. The Cgctr2 mutants had markedly reduced Cu superoxide dismutase (SOD) activity, suggesting that CgCTR2 is important in providing copper to copper-dependent cytosolic activities. The Cgctr2-silenced mutants had increased sensitivity to H$_2$O$_2$ and reduced germination rates. The mutants were also less virulent to plants, but they did not display any defects in appressorium formation and penetration efficiency. An external copper supply compensated for the hypersensitivity to H$_2$O$_2$ but not for the germination and pathogenicity defects of the mutants. Similarly, overexpression of CgCTR2 enhanced resistance to H$_2$O$_2$ but had no effect on germination or pathogenicity. Our results show that copper is necessary for optimal germination and pathogenicity and that CgCTR2 is involved in regulating cellular copper balance during these processes.

Spores of plant-pathogenic fungi can germinate in water, unlike spores of “saprophytic” species, which usually germinate in the presence of sugars (8, 17). In many cases germination is stimulated by specific plant compounds or by physicochemical properties of the supporting surface. In *Colletotrichum* species cuticular waxes or plant volatiles can induce germination (19, 26). Spore germination in soil-borne fungi is enhanced by root exudates, while in rust fungi spores are sensitive to the leaf surface properties (2, 12). Hard hydrophobic surfaces can replace plant-derived signals and are sufficient to trigger spore germination in several species, including *Magnaporthe grisea*, *Botrytis cinerea*, and *Colletotrichum* species (7, 8, 11, 15).

Spore germination is regulated by different signaling cascades. In most species heterotrimeric G proteins, cyclic AMP (cAMP), and mitogen-activated protein (MAP) kinase cascades are involved in the activation of germination as well as in the regulation of specific developmental stages during germination. In *Colletotrichum lagenarium* germination is controlled by at least two signaling cascades, a cAMP-dependent pathway and the CMK1 MAP kinase (homolog of *M. grisea* PMK1) pathway (29). Additional signaling elements might be involved, e.g., primarily calcium-dependent signals and other MAP kinase pathways (11, 13, 27). Germination in the gray mold fungus *B. cinerea* is regulated by at least three signaling pathways, which include a Gα protein BCG3, a CAMP pathway, and the FUS3/PMK1 MAP kinase homolog BPM1 (8). Each of these pathways mediates germination in response to different signals including carbon source, surface hardness and hydrophobicity, or specific nutrients. Spore germination in *M. grisea* does not require the PMK1 or Gα/CAMP pathways; however, these pathways regulate the following formation of appressoria, which are specialized organs that differentiate at the end of germ tubes and are used for plant penetration (31, 33). Moreover, 19 out of 67 genes that were highly expressed in appressoria compared to mycelium also showed high levels in dormant spores (30). Thus, in plant-pathogenic fungi, spore germination and early pathogenic development are tightly linked and are regulated by common signaling pathways.

*Colletotrichum gloeosporioides* f. sp. *aeschynomene* is a hemibiotrophic plant pathogen that specifically attacks the weed *Aeschynomene virginica*. The fungus produces large numbers of asexual spores that, following a contact with plant organs, germinate, form appressoria, and penetrate the plant. We pre-
viously showed that \textit{C. gloeosporioides} can germinate in two distinct ways: “pathogenic” and “saprophytic” (3). Pathogenic germination takes place on plants or on a hydrophobic surface and is characterized by fast mitosis followed by development of a single germ tube. The process is initiated immediately following induction and terminates within 4 h with the formation of appressoria. Saprophytic germination occurs in rich medium; it takes a much longer period of time and is characterized by development of two germ tubes that emerge from opposite sides of the spore. These germ tubes do not form appressoria, and therefore spores that germinate in this way do not infect plants. The two germination styles in \textit{C. gloeosporioides} are regulated by different signaling cascades; saprophytic germination is enhanced by cAMP while pathogenic germination is cAMP independent, but, similar to \textit{M. grisea}, cAMP is required later for appressorium formation (3). The association between germination style and the subsequent pathogenic development suggests that genes that are necessary for pathogenic spore germination may also affect fungal pathogenicity.

To identify genes that are associated with pathogenic germination, we generated a \textit{C. gloeosporioides} promoter-trapping mutant collection by restriction enzyme-mediated transformation (REMI), using the green fluorescent protein (GFP) as a reporter for gene expression. We screened this collection for transformants that had specific GFP expression patterns under saprophytic and pathogenic germination conditions. Here, we report on the characterization of a REMI mutant in which GFP expression was enhanced in spores and suppressed during pathogenic germination. The tagged locus was isolated and found to encode a putative vacuolar copper transporter that has not been previously characterized in filamentous fungi. Functional analysis of this gene revealed that it is involved in proper germination and pathogenesis in this fungus.

\section*{MATERIALS AND METHODS}

\subsection*{Fungi and plants.} \textit{C. gloeosporioides} f. sp. \textit{aeschynomene} strain 3.1.3 and transgenic strains were used. Emerson’s YPSS (EMS), pea extract (PE), regeneration (REG) and Czapek-Dox (CD) solid and liquid media were prepared as previously described (25). Pathogenicity tests were performed on 12-day-old \textit{Aeschynomene vagnica} plants or on the first true leaves of \textit{Psam aestivum} cv. white sugar (3). \textit{M. grisea} wild-type strain 70-15 and transgenic strains were cultured on oatmeal agar plates at 25°C under fluorescent light (32).

\subsection*{Generation of REMI collection.} Plasmid pAS1 (provided by W. Schäfer) was digested with NcoI and SpflI and the \textit{EGFP} (where EGFP is enhanced green fluorescent protein) promoter-trapping vector was electroporated with spores (26) with 1 \textmu g of plasmid DNA.

\subsection*{Construction of plasmid vectors.} (i) Ksh-CgCTR: CgCTR2 silencing vector. Fragments from bp 1 to 700 and 1 to 1024 of the CgCTR2 genomic sequence were amplified by PCR using the primer pair Dw-1Bm (CCCAAGGACCATGACCGACCGGTGATA) and Up-716sp (GTTCCTAGTACATGCGGCGGACCACCTGCCAGACCG) and the pair Up-1NcoI (CCCAAGAACCACCATGACCGACCGGTGATA) and Dw-1024sp (CCCAAGAACCACCATGACCGACCGGTGATA) respectively. The 1,024-bp fragment was cloned into NcoI and SpeI sites of the \textit{PGEM} (Promega). The 700-bp fragment was cloned into SacI and SpeI sites of the resulting plasmid. The two fragments in opposite orientations were released by digestion with NcoI and BamHI and cloned into the \textit{PGEM} promoter and \textit{TRPC} terminator in pKsh52-1 (4).

(ii) \textit{Ksh-CgCTR}: CgCTR2 overexpression vector. The full-length CgCTR2 genomic clone (1.2 kb; accession number EF434817) was amplified by PCR with the primers 159oe-Bgl (GAATGGGGAGATCTTTCAATGGCAGGCGTTG) and 159oe-Bsp (GGGGGTCATGATTGAATGGCAGGCGTGTG) and cloned into pT7Z5RT (Ferments). The 35S-GFP-CTR2 promoter was amplified by PCR using the primers 159oe-Bgl (see Ksh-CgCTR) and 159spf-for (TGTCGTAGATCATTGAGGCGGACCACCGGTGATA) and cloned into BspHI and BglII sites of the \textit{PGEM} promoter and \textit{TRPC} terminator in pKsh52-1.

(iii) \textit{p57-EGFP-CTR2} vector. The 3-fragment-kb genomic fragment including the CgCTR2 promoter (1.8 kb) and open reading frame (ORF; 1.2 kb) was amplified by PCR using the primers 159oe-Bgl (see Ksh-CgCTR) and 159spf-for (TGTCGTAGATCATTGAGGCGGACCACCGGTGATA). The fragment was cloned into pT7Z5RT (Ferments).

(iv) \textit{pGm10-CTR2} vector. The \textit{pGm10-CTR2} vector was amplified with primers 159oe-Bgl (see Ksh-CgCTR) and \textit{CTR}fusion-BsphI (GGGGGTCATGATTGAATGGCAGGCGTGTG) and cloned into an NcoI site on \textit{KshCFP} (where CFP is cyan fluorescent protein). The resulting \textit{cDNA} were cloned into BamHI and KpnI sites under the \textit{GALT} promoter in pGm10-T.

(v) \textit{CgCTR2-CFP}. The \textit{CgCTR2-CFP} expression vector was amplified with primers 159oe-Bgl (see Ksh-CgCTR) and \textit{CTR}fusion-BsphI (GGGGGTCATGATTGAATGGCAGGCGTGTG) and cloned into an NcoI site on \textit{KshCFP} (where CFP is cyan fluorescent protein). The resulting \textit{cDNA} were cloned into BamHI and KpnI sites under the \textit{GALT} promoter in pGM10-T.

(vi) \textit{pH14107-\textit{MgCTR2}} vector. The full-length \textit{M. grisea} \textit{CTR2} (\textit{MgCTR2B}; Broad accession no. MG00548) gene was amplified with primers \textit{CTR}PBF (ATATGCCGCGCAGCGACAGTATGAATGGCAGGCGTGTG) and \textit{CTR}PRF (ATATGCCGCGCAGCGACAGTATGAATGGCAGGCGTGTG) and cloned into the \textit{Nol} and \textit{HindIII} sites on \textit{pYK11}, which has a blomycin resistance cassette (33).

\subsection*{Yeast complementation assay.} \textit{ctr1Δ, ctr2Δ, and ctr3Δ yeast cells} were transformed with the plasmids \textit{pRS416, pRS416-\textit{CTR2}, pGMT10-\textit{CgCTR2S}}, and \textit{pGm101-\textit{CgCTR2L}}. Two transformants from each transformation were selected and grown to exponential phase in medium without uracil. Cells were collected by centrifugation, washed with sterile distilled water, and diluted with water to an optical density at 600 nm of 1. Serial dilutions were spotted on selective medium (yeast extract and peptone with 2% ethanol and 3% glycerol [\textit{YPEG}]) and on \textit{YPEG} medium with 10, 15, 20, 25, and 50, and 100 \mu M \textit{CuCl}2. Plates were incubated at 30°C for 3 to 7 days. \textit{CgCTR2} localization. Transgenic isolates expressing the \textit{CgCTR2-CFP} fusion cassette were examined using a laser scanning confocal microscope (Zeiss LSM 510). Staining with \textit{FM4-64} and \textit{DMY-64} (Molecular Probes) was performed according to the manufacturer’s instructions.

\subsection*{Superoxide dismutase (SOD) activity assay.} Fungi were cultured in CD medium for 48 h, and then the mycelium was harvested. Dry hyphalipid mycelia were crushed to powder and suspended in lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA). The samples were centrifuged at 4°C at maximum speed for 10 min. The supernatant was then transferred into new tubes, and the protein concentration was determined. Protein samples (10 \mu g) were separated on a 12.5% nondenaturing polyacrylamide gel. Gels were incubated for 30 min in the dark in 40 ml of reaction mixture containing 0.1 M potassium phosphate buffer, pH 7.8, 1 mM EDTA, 33 \mu M riboflavin (Sigma-Aldrich), 245 \mu M nitroblue tetrazolium (Sigma-Aldrich), and 17 mM TEMED (\textit{N,N,N’,N’}-tetramethylethylenediamine). The gels were run at 4°C at maximum speed for 1 h. The stain was then transferred into new tubes, and the protein concentration was determined. Protein samples (10 \mu g) were separated on a 12.5% nondenaturing polyacrylamide gel. Gels were incubated for 30 min in the dark in 40 ml of reaction mixture containing 0.1 M potassium phosphate buffer, pH 7.8, 1 mM EDTA, 33 \mu M riboflavin (Sigma-Aldrich), 245 \mu M nitroblue tetrazolium (Sigma-Aldrich), and 17 mM TEMED (\textit{N,N,N’,N’}-tetramethylethylenediamine). The gels were run at 4°C at maximum speed for 1 h. The stain was then transferred into new tubes, and the protein concentration was determined.
phosphotransferase) cassette. The MgCTR2 gene replacement construct was amplified with primers CTR1F and CTR4R using the ligation product as the template and directly transformed into M. grisea strain 70-15. Hygromycin-resistant colonies were screened by PCR with primers CTRNF (CCAGAGGAGCTAGCTGTCAGAGC) and CTR2Rspecific primers (159-start and 159end-new) was performed using 4 ng of cDNA as a template. RT-PCR, cDNA was generated by genome walking. Analysis of the isolated DNA sequence revealed that the pAS-GFP vector was inserted 70 bp upstream of a putative ORF (Fig. 2). Generation of cDNA with primers corresponding to the 5’ and the 3’ ends of the putative ORF yielded two DNA fragments corresponding to 504-bp and 549-bp transcripts. RT-PCR with a primer corresponding to the 3’ end of the gene and a primer matching the 45-bp region that was found only on the larger transcript yielded a single fragment, confirming that both transcripts are encoded by this locus. Comparison of the genomic and cDNA sequences showed that the gene has three introns and that the difference between the two cDNAs is in the third exon (Fig. 2). A BLASTP search with both predicted proteins (183 amino acids [aa] and 168 aa) as a query showed significant similarity to a putative copper transporter from Candida albicans.

**RESULTS**

**Characterization of REMI mutant N159.** C. gloeosporioides strain 3.1.3 was transformed with the NeoI-digested plasmid pAS-GFP, which includes a promoterless EGFP gene adjacent to the NeoI restriction site. Spatial and temporal GFP expression patterns were determined in individual strains that were randomly chosen from more than 4,000 transformants in the REMI collection. In strain N159 bright GFP expression was observed in spores on EMS plates. Further characterization of strain N159 revealed differences in GFP expression under several growth conditions. An intense GFP signal was detected in all organs on high-osmotic medium (REG), whereas on EMS medium the GFP signal was strong in spores and weak in hyphae (Fig. 1A). The GFP signal was hardly detected when the fungus was grown in PE medium and was recovered when the mycelium was transferred from PE to REG medium (Fig. 1B), suggesting that the EGFP gene was specifically downregulated in PE medium. The N159 strain had normal morphology on agar plates but had reduced germination compared to the wild-type strain. In a typical assay on glass slides 50% (± 8%) of N159 spores germinated after 2.5 h, compared with 87% (± 4%) germination in the wild-type 3.1.3 strain (Table 1).

**Isolation of the tagged locus in strain N159.** Southern blot analyses confirmed a single insertion of the transformation vector in N159 (data not shown). DNA fragments flanking the insertion site were recovered by inverse PCR, and additional DNA sequences were generated by genome walking. Analysis of the isolated DNA sequence revealed that the pAS-GFP vector was inserted 70 bp upstream of a putative ORF (Fig. 2). Generation of cDNA with primers corresponding to the 5’ and the 3’ ends of the putative ORF yielded two DNA fragments corresponding to 504-bp and 549-bp transcripts. RT-PCR with a primer corresponding to the 3’ end of the gene and a primer matching the 45-bp region that was found only on the larger transcript yielded a single fragment, confirming that both transcripts are encoded by this locus. Comparison of the genomic and cDNA sequences showed that the gene has three introns and that the difference between the two cDNAs is in the third exon (Fig. 2). A BLASTP search with both predicted proteins (183 amino acids [aa] and 168 aa) as a query showed significant similarity to a putative copper transporter from Candida albicans.

**TABLE 1. Spore germination in CgCTR2 transgenic strains**

<table>
<thead>
<tr>
<th>Strain*</th>
<th>% Germination (± SD)b</th>
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<tr>
<td>3.1.3 (wild type)</td>
<td>87 ± 4</td>
</tr>
<tr>
<td>N159</td>
<td>58 ± 15</td>
</tr>
<tr>
<td>RNAi 9</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>RNAi 49</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>OCX35</td>
<td>85 ± 3</td>
</tr>
<tr>
<td>OCX29</td>
<td>83 ± 6</td>
</tr>
<tr>
<td>C10</td>
<td>88 ± 2</td>
</tr>
<tr>
<td>C21</td>
<td>89 ± 2</td>
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</table>

*OX35 and OC29 are CgCTR2 overexpression strains. C10 and C21 are CgCTR2 complementation strains.

b Results are the percentage of spores (10⁶ conidia/ml) that germinated after 2.5 h.
moral, which lacks the high affinity (CTR1 vacuole) copper transporter genes. The triple \(incerH9004\) double mutant, which can grow normally on YPEG medium (vector).

Genome searches revealed a single predicted protein with significant homology to CgCTR2 in each of the filamentous fungi for which a genome sequence is available (see Fig. S1 in the supplemental material). Copper transporters are characterized by three putative transmembrane domains and several MXM, MXXM, or MXXXM (where X is any other amino acid) methionine motifs (21). The long and short predicted peptides of CgCTR2 have two and three transmembrane domains, respectively, and each peptide contains four methionine motifs. These analyses suggested that CgCTR2 encodes a copper transporter of the vacuolar membrane.

Functional complementation of \(S. ceriseiae\) CTR mutants. Yeast \(ctr\) mutants were complemented with the CgCTR2 gene. The long and short CgCTR2 transcripts were expressed under the \(GAL\) promoter in an \(S. ceriseiae\) \(ctr1Δctr2Δctr3Δ\) triple mutant, which lacks the high affinity \((CTR1 and CTR3)\) and vacuole \((CTR2)\) copper transporter genes. The triple \(ctr\) yeast mutant is unable to grow on ethanol-glycerol (YPEG) medium unless supplemented with >15 \(\muM\) copper, unlike the \(ctr1Δ\) \(ctr3Δ\) double mutant, which can grow normally on YPEG medium with 15 \(\muM\) copper (23). Expression of CgCTR2 in the \(S. ceriseiae\) \(ctr1Δctr2Δctr3Δ\) mutant strain fully restored growth on YPEG medium with 15 \(\muM\) CuCl\(_2\) (Fig. 3), further suggesting that CgCTR2 is a functional homologue of the \(S. ceriseiae\) Ctr2 vacuolar copper transporter.

\(CgCTR2\) localization. To determine the localization of the CgCTR2 protein, we generated transgenic isolates expressing a CgCTR2-CFP fusion transcript. The cyan signal originating from the fusion protein was detected inside the cells, either as clear circles or as spots (Fig. 4). No signal could be detected on the outer cell membrane, suggesting that CgCTR2 is strictly localized inside the cell. Staining with the vacuole membrane-specific marker MDY-64 gave a similar staining pattern (Fig. 4A, frame c). Dual staining with both CFP and MDY-64 was not possible due to the overlapping of the excitation and emission spectra. In order to rule out possibility that the CFP was localized in nuclei, we compared Hoechst-stained nuclei with differential interference contrast (DIC) images (Fig. 4B). Vacuoles are clearly seen in the DIC images, and the overlay shows that nuclei are located between these large vacuoles.

Although CFP signal surrounded the nuclei (open circles in Fig. 4A, frame a, and C, frames c and h), most of the CFP signal was detected inside large vacuoles or on the membranes of small vacuoles (Fig. 4C). Staining CgCRT2-CFP transgenic hyphae for a short period of time with FM4-64, which stained the cell plasma membrane, confirmed that the CFP signal was not localized in the cell plasma membrane (Fig. 4D, frames a and b). Staining for a longer period with FM4-64 resulted in FM4-64-labeled intracellular membranes that colocalized with the CFP signal (Fig. 4D, frames c, d, and e). These results show that CgCTR2 is localized inside the cell in vacuoles and on vacuole membranes.

\(CgCTR2\) gene expression. Expression of CgCTR2 was determined under various culture conditions, during germination, and following plant inoculation.

\(Copper\). Response to copper was determined by exposing mycelia to 0, 10, 20, 100, and 250 \(\muM\) concentrations of CuCl\(_2\). Expression of CgCTR2 was highest on a medium without copper and slightly reduced by 10 and 20 \(\muM\) CuCl\(_2\). No further changes were observed at higher copper concentrations (Fig. 5A).

\(Media\). To determine the effect of different media on CgCTR2 gene expression, we compared expression of CgCTR2 in mycelia that were produced in REG medium (a high osmotic, rich medium) and in PE or EMS medium, which induce pathogenic and saprophytic germination, respectively (3). Strongest expression was detected in spores and mycelia that were produced in REG (mycelium) and EMS (spores and mycelium) media whereas in PE medium the signal was below the detection level (Fig. 5B). These results are in agreement with the GFP expression pattern in strain N159, which was most intense in REG medium and in spores and hardly detected in PE medium (Fig. 1). To rule out the possibility that downregula-
tion of the gene was possibly due to high copper levels in PE medium, we grew the N159 strain in PE medium supplemented with 5 and 100 μM copper or with the copper chelator EGTA. Under all of these conditions, expression of GFP was not altered and remained extremely low, indicating that the gene was repressed by components of the PE medium (data not shown).

Germination. CgCTR2 gene expression was followed in spores germinated in PE or EMS medium. In resting spores, expression of CgCTR2 was strong, similar to the GFP signal observed in spores produced by N159 on EMS plates. In EMS medium expression was stable throughout the entire germination process, whereas in PE medium CgCTR2 was downregulated already after 0.5 h, and no transcript could be detected thereafter (Fig. 5C).

In planta. Pea leaves were inoculated with spores, and tissue samples were collected from the infected area at several time points following inoculation. RNA was extracted from the infected plant tissues, and the relative expression of CgCTR2 was determined by quantitative RT-PCR. Expression of CgCTR2 was most intense in spores at time zero and was reduced to below detection levels immediately following plant inoculation, with no transcript detectable until 48 h postinoculation (Fig. 5D). A moderate level of CgCTR2 gene expression was recovered 72 h postinoculation.

The apparent expression pattern of CgCTR2 indicates that it is highly expressed in resting spores and strongly repressed at the onset of pathogenic development.

Generation of CgCTR2 transgenic strains. Insertion of the transformation cassette in strain N159 was at the 5' untranslated region, close to the ATG, leaving the reading frame intact. RT-PCR analysis showed that CgCTR2 transcript levels were significantly reduced in strain N159 but not completely abolished (Fig. 6A). We used RNA interference (RNAi) to cause silencing of CgCTR2. Complete silencing of CgCTR2 was obtained in RNAi strains 9 and 49 (Fig. 6A). CgCTR2-overexpressing isolates were produced by expression of the gene from the Aspergillus nidulans GPDA promoter. CgCTR2 expression in these isolates was high under all conditions, including in PE medium, in which the CgCTR2 transcript is normally undetected (Fig. 6B). The N159 mutant strain was also transformed with a CgCTR2 complementation vector containing the CgCTR2 promoter and ORF. Analyses were car-

FIG. 4. CgCTR2 is localized in the vacuole membrane. Transformants expressing a CgCTR2-CFP fusion protein were visualized using confocal and fluorescent microscopes. (A) Confocal images of CFP and DMY-64 signals. Different samples are shown due to the overlap of the spectra between CFP and DMY-64. Frames a and b, CFP and DIC images; frames c and d, DMY-64 and DIC images. (B) Fluorescent microscopy of Hoechst-stained nuclei and DIC images. Frame e, Hoechst staining; frame f, DIC image (white arrow, vacuole; black arrow, nucleus); frame g, merge. (C) Fluorescent microscope images of CFP and Hoechst staining. Hyphae containing large (left) or small (right) vacuoles are shown. Frames a and b, DIC images; frames c and d, CFP (white arrows show large vacuoles; yellow arrows show small vacuoles); frames e and f, Hoechst staining; frames g and h, merged images of CFP and Hoechst staining. (D) Confocal images of FM4-64 and CFP signals. Frames a and b show the results of a short incubation with FM4-64. The FM4-64 signal (a) is detected on the outer cell membrane; CFP signal (b) is inside the cell; Frames c, d, and e show results of longer incubations with FM4-64; frame c, CFP; frame d, FM4-64; frame e, merged image. Membranes of large vacuoles are stained with FM4-64; CFP signal is localized inside these vacuoles as well as around nuclei. Scale bar, is 5 μm.
Sensitivity of the CgCTR2 strains to copper and hydrogen peroxide. Sensitivity to copper and oxidative conditions was determined by measuring the effect of CuCl₂ and hydrogen peroxide (H₂O₂) on radial growth. The addition of copper to the medium had the same effect on the growth rates of the wild-type and transgenic strains; concentrations of 1 mM CuCl₂ or higher were lethal to all strains, whereas at lower copper concentrations the mutant and CgCTR2 overexpression strains had the same growth rates as the wild type (data not shown). The addition of iron instead of copper had no effect on the wild type or the mutants (data not shown). Thus, the hypersensitivity of ctr2 mutants to H₂O₂ can be specifically restored by an external supply of copper.

Cu/Zn SOD activity in Cgctr2 mutants. To test the postulated role of CgCTR2 in transporting copper to cytosolic copper-dependent enzymes, we measured the activity of the cytosolic Cu/Zn SOD enzyme, which requires copper for activity. Wild type and the RNAi 7 and N-159 mutant strains were grown in CD medium which does not contain copper. Mycelium was harvested after 48 h, and SOD activity was determined. Without copper, the wild type had an extra band that could not be detected in the mutants (Fig. 7). This band was intensified and was also detected in the mutants when copper was reintroduced with complementation strains C10 and C37, which expressed normal levels of CgCTR2 (data not shown).

Several yeast species in which ctr2 mutants are unaffected in growth in response to copper but are hypersensitive to H₂O₂ (5, 16). In order to determine whether the hypersensitivity of the mutants to H₂O₂ was due to copper deficiency, we compared the growth rates of the strains on H₂O₂-containing medium supplemented with copper. The addition of low levels of copper (5 to 50 nM) had no effect on the growth rate of the wild type in H₂O₂-containing medium, but the same concentrations of copper greatly improved the growth rates of the mutants and restored the rates to wild-type levels (Table 3). The addition of iron instead of copper had no effect on the wild type or the mutants (data not shown). Thus, the hypersensitivity of Cgctr2 mutants to H₂O₂ can be specifically restored by an external supply of copper.

### TABLE 2. Sensitivity of CgCTR2 transgenic strains to H₂O₂

<table>
<thead>
<tr>
<th>Strain</th>
<th>Radial growth (cm) at the indicated H₂O₂ concn (mM)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>3.1.3 (wild type)</td>
<td>2.62</td>
</tr>
<tr>
<td>N159</td>
<td>2.44</td>
</tr>
<tr>
<td>RNAi 9</td>
<td>2.59</td>
</tr>
<tr>
<td>OX29a</td>
<td>2.59</td>
</tr>
<tr>
<td>C10b</td>
<td>2.60</td>
</tr>
</tbody>
</table>

*a Values were determined after 5 days of incubation under dark conditions. Results are the means of four replications. The standard deviation was <0.1.

b OX29 is a CgCTR2 overexpression strain.

c C10 is a CgCTR2 complementation strain.
was added. These results show that under copper-limiting conditions, CgCTR2 is essential for supplying the copper required for the activity of a copper-dependent cytosolic enzyme. Two additional SOD activity bands, which were unaffected by copper, were observed under all conditions. These could be associated with Mn SOD.

**Spore germination in Cgctr2 mutants.** Spores (10^6/ml) of the RNAi strains had a reduced ability to germinate, similar to germination of N159 (Table 1). The germination defects of the Cgctr2 mutants were more pronounced at high spore densities. Less than 50% of Cgctr2 mutant conidia germinated at 2 x 10^6 or 10^7 spores/ml, while at lower spore densities germination rates were increased and reached nearly wild-type levels at or below 10^5 spores/ml (Fig. 8). Germination in the CgCTR2 overexpression and complementation strains was unchanged compared to the 3.1.3 wild-type strain (Table 1). Copper enhanced spor germination in both the Cgctr2 mutants and the wild-type strain. For the wild-type and N159 strains the germination rates in PE medium alone were 74% and 39% ± 4%, respectively. With the addition of copper (100 μM CuCl₂), the germination rates were 87.6% ± 2% for the wild type and 54.8% ± 5% for strain N159. Thus, an external copper supply can enhance germination, but this phenomenon is unrelated to CgCTR2.

**Pathogenicity of the Cgctr2 mutants.** When sprayed (5 x 10^4 spores/ml) on A. virginica plants, the Cgctr2 mutant strains (N159, RNAi 9, and RNAi 49) caused delayed and reduced symptoms compared with plants infected by the 3.1.3 wild-type strain. The level of plant mortality 6 days postinoculation was always lower than 50% in mutant-infected plants, compared to nearly 100% mortality in wild-type-infected plants (Fig. 9A). The complementation and overexpression strains had wild-type pathogenicity.

An onion epidermis penetration assay and inoculation of detached pea leaves were used to characterize the early stages of infection by the mutants. Since the RNAi and N159 strains had similar phenotypes, we used only the N159 strain in these experiments because it carried the GFP marker suitable for detection of in planta growth. There was no difference between N159 and the gGFP strain in appressorium formation and penetration on onion epidermis. In vitro assays of appressorium formation in petri dishes confirmed that the N159 strain was unaffected in appressorium formation (data not shown). Detached pea leaves developed similar symptoms when infected with spores of N159 or the gGFP-expressing strains having wild-type pathogenicity, further demonstrating that the Cgctr2 mutants form fully functional appressoria (Fig. 9B). Thus, the CgCTR2 gene is dispensable for appressorium formation and function.

Usually, the fungus will penetrate the plant several hours after appressorium formation, and further development will occur inside the plant tissues (3, 4). Extensive hyphal growth on the plant surface was not observed in the wild-type strain 24 h postinoculation. In contrast, spores of strain N159 produced abundant hyphae on the leaf surface (Fig. 9B). The development of hyphae on the leaf surface has been previously correlated with saprophytic germination, in which germ tubes develop into long hyphae that do not differentiate appressoria (3).

Infection of A. virginica plants by C. gloeosporioides increases with increasing numbers of spores and reaches saturation at or near 5 x 10^6 spores/ml. Higher numbers of spores do not cause a significant increase in disease symptoms or plant mortality. The Cgctr2 mutants developed fully functional appressoria but had lower rates of pathogenic germination, which might affect the number of spores that can initiate infection. To determine whether low rates of pathogenic germination might cause the reduced virulence of Cgctr2 mutants, we compared disease

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**TABLE 3. Copper compensation for hypersensitivity of the Cgctr2 mutant to H₂O₂**

<table>
<thead>
<tr>
<th>CuCl₂ concn (nM)</th>
<th>Radial growth (cm) of the indicated straina</th>
<th>% Germination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without H₂O₂</td>
<td>With H₂O₂ (1.6 mM)</td>
</tr>
<tr>
<td>0</td>
<td>2.20 ± 0.05</td>
<td>2.11 ± 0.03</td>
</tr>
<tr>
<td>50</td>
<td>2.11 ± 0.02</td>
<td>2.06 ± 0.02</td>
</tr>
</tbody>
</table>

a Growth was determined after 5 days of incubation under dark conditions. Values are the means of four replications ± standard deviations.

**FIG. 8.** Effect of spore density on germination rate. Spores of the wild-type (■) and N159 (□) strains were collected from EMS plates, diluted, and germinated in PE medium. After 2.5 h the number of germinated spores was counted, and the percentage of germination was calculated. Germination in the mutant strains was markedly reduced compared to the wild type at spore densities of 10⁵/ml or higher but was similar to the wild type at or below 10⁵ spores/ml.

**FIG. 7.** Activity assay of Cu/Zn SOD in wild-type and Cgctr2 mutant strains. Strains were grown in CD medium without copper for 48 h. Mycelium was harvested, and SOD activity was determined with or without the addition of 5 μM copper. Without copper the wild type showed an additional band (arrow) that could not be detected in the RNAi and N159 mutant strains. This band was intensified and was present in all strains following the addition of 5 μM copper. The other two bands were unchanged under all conditions and might represent the activity of Mn SOD. WT, wild type.
levels at high (8 × 10^4) or low (2 × 10^4) numbers of spores with disease levels caused by 5 × 10^4 spores/ml. When plants were inoculated with 8 × 10^3 spores/ml, the symptoms caused by the Cgctr2 mutant strains were enhanced compared to infection with 5 × 10^4 spores/ml and were similar to symptoms caused by the 3.1.3 wild-type strain (Fig. 9C). When the number of spores was reduced to 2 × 10^3/ml, infection rates of both the wild type and N159 were reduced in comparison to infection with 5 × 10^4 spores/ml. The symptoms caused by 2 × 10^3 spores/ml of the wild-type strain were similar to the symptoms that were caused by 5 × 10^4 spores/ml of strain N159. That the reduced virulence of the Cgctr2 mutants could be compensated for by increasing the number of spores suggests that the low rates of spore germination among the mutants might contribute to their reduced virulence.

The MgCTR2 gene in M. grisea is a functional homolog of CgCTR2. A single homolog of CgCTR2, MGF00548, was identified in the M. grisea genome (named MgCTR2). Three transformants of strain N159 carrying the MgCTR2 gene (entire ORF plus its native promoter) were generated and confirmed by Southern analysis (data not shown). Germination in N159::MgCTR2 strains was between 70% and 75%, significantly higher than 47% germination in N159 but somewhat lower than the 83% germination in the C. gloeosporioides wild-type strain 3.1.3 (Table 4). The hypersensitivity of Cgctr2 mutants to H_2O_2 was fully rescued by MgCTR2 (Table 4), indicating that MgCTR2 functionally complemented the C. gloeosporioides Cgctr2 mutant. Partial restoration of the germination defects in N159::MgCTR2 transformants might be related to the expression level of the MgCTR2 promoter in C. gloeosporioides.

To determine the function of MgCTR2 in M. grisea, we generated an Mgctr2 deletion mutant (see Materials and Methods). When assayed by vegetative growth on agar plates, the Mgctr2 mutant (strain CTR-23) showed increased sensitivity to H_2O_2 in comparison with the ectopic (CTR-18) transformant (Table 5). Spore germination was less sensitive to H_2O_2 than mycelium growth in the Mgctr2 mutant (Table 5). However, appressorium formation and melanization were highly sensitive to oxidative stress. As low as 0.8 mM H_2O_2 was effective in reducing appressorium formation, and no appressorium formation was observed in the Mgctr2 mutant in the presence of 3.2 mM H_2O_2. The increased sensitivity of the Mgctr2 mutant to oxidative stress further confirmed our earlier observations with the RNAi silencing transformants in C. gloeosporioides and showed that the function of MgCTR2 in M. grisea was similar to that of CgCTR2 in C. gloeosporioides.

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Germination</th>
<th>Radial growth (cm) at the indicated H_2O_2 concn (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>3.1.3</td>
<td>83 ± 4.0</td>
<td>1.87</td>
</tr>
<tr>
<td>N159</td>
<td>47.8 ± 3.7</td>
<td>1.83</td>
</tr>
<tr>
<td>MgCTR2-1</td>
<td>70.5 ± 4.8</td>
<td>1.90</td>
</tr>
<tr>
<td>MgCTR2-3</td>
<td>69 ± 5.1</td>
<td>1.80</td>
</tr>
</tbody>
</table>

*a MgCTR2-1 and MgCTR2-3: strain N159 complemented with M. grisea MgCTR2, isolates 1 and 3, respectively. Similar results were obtained with additional isolates.

*Values are the means of four replications ± standard errors. All standard errors were < 0.05.

*Growth was determined after 5 days of incubation under dark conditions.
TABLE 5. H$_2$O$_2$ sensitivity of Mgcctr2 mutants$^a$

<table>
<thead>
<tr>
<th>H$_2$O$_2$ concen (mM)</th>
<th>$%$ Growth</th>
<th>% Germination</th>
<th>% Appressorium formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTR-18</td>
<td>CTR-23</td>
<td>CTR-18</td>
</tr>
<tr>
<td>0</td>
<td>4.0 ± 0.2</td>
<td>4.0 ± 0.2</td>
<td>96.1 ± 6.3</td>
</tr>
<tr>
<td>0.8</td>
<td>3.8 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>95.2 ± 8.1</td>
</tr>
<tr>
<td>1.6</td>
<td>3.5 ± 0.2</td>
<td>3.4 ± 0.1</td>
<td>92.3 ± 7.8</td>
</tr>
<tr>
<td>3.2</td>
<td>3.0 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>87.8 ± 9.1</td>
</tr>
<tr>
<td>6.25</td>
<td>1.6 ± 0.1</td>
<td></td>
<td>63.9 ± 6.7</td>
</tr>
<tr>
<td>12.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ CTR-18, ectopic transformant; CTR-23, Mgcctr2 deletion strain. Radial growth was determined after 5 days of incubation under dark conditions. Values are the means four replications ± standard errors.

**DISCUSSION**

We previously showed that germination in *C. gloeosporioides* can follow two different routes, which are associated with pathogenic or saprophytic lifestyles (3). In the pathogenic mode, cell cycle and morphogenesis take place immediately after exposure to inducing signals, while in the saprophytic mode germination starts only several hours after induction. It is therefore suggested that pathogenic germination depends on genes (and proteins) that are expressed in the resting spores and can activate morphogenesis and the cell cycle upon receiving an appropriate signal. In this study, we exploited a collection of transgenic strains that were generated with a plasmid containing a GFP reporter gene to identify genes that are specifically related to pathogenic germination.

In strain N159, the GFP signal was intense in resting spores and was specifically repressed during pathogenic germination. The tagged locus was isolated, and the CgCTR2 gene, which is located 70 bp downstream of the insertion site in strain N159, was characterized. The predicted CgCTR2 protein shares high identity with *S. cerevisiae* Ctr2p, a copper transporter of the vacuolar membrane. The *S. cerevisiae* Ctr2p is a member of the CTR family of integral membrane proteins that function in copper uptake (1). The high-affinity copper transporter protein members Ctr1p and Ctr3p are localized in the plasma membrane and import copper into the cell. These proteins have been studied in various organisms and are the primary transporters of external copper into the cell. A third member of the CTR family of proteins (Ctr2p) was studied in only a few species. It was initially identified by homology to the plant copper transporter Copt1 and was suggested to be a low-affinity copper transporter due to lack of a clear phenotype in yeast and *Podospora anserine* ctr2Δ mutants (6, 10). However, more recent studies showed that Ctr2p is localized to the vacuole membrane and that it is involved in regulation of the intracellular copper concentration by exporting copper from the vacuole into the cytosol (23). Localization of the *Arabidopsis* and *P. anserine* homologs is unknown, but they might well represent vacuolar transporters.

The redox sensitivity of copper makes it an essential cofactor in critical biological processes such as respiration, iron transport, oxidative stress protection, and pigmentation. *S. cerevisiae* ctr1Δ ctr3Δ ctr2Δ triple mutants are unable to grow on ethanol-glycerol medium (YPEG) due to insufficient delivery of copper to cytochrome c oxidase. The strain can grow only on YPEG medium containing relatively high levels of copper. This defect is partially compensated for by complementation of the triple ctrΔ mutant with the wild-type CTR2 gene (23). *S. cerevisiae* ctr1Δ ctr3Δ ctr2Δ expressing CgCTR2 (either the long or short transcript) was unable to grow on YPEG medium without copper but exhibited improved growth on YPEG medium with a low copper concentration, which was even better than the growth of the same strain complemented with *S. cerevisiae* CTR2 (Fig. 3). Further, a CgCTR2::CFP fusion protein was localized in vacuoles (Fig. 4), similar to yeast Ctr2p (23). In large vacuoles the signal was detected inside the vacuoles, whereas in small vacuoles it was detected on the vacuole membrane. The localization inside large vacuoles could be the result of the overexpression or be due to the internalization of the protein following inactivation of these vacuoles. Importantly, CgCTR2 could not be detected in the cell plasma membrane, thus ruling out the possibility that this protein is similar to CTR1 or CTR3 and involved in copper uptake. Together, the sequence homology, functional analysis, and localization data strongly suggest that CgCTR2 is a vacuole copper transporter.

Yeast CTR2 encodes a single peptide, whereas the CgCTR2 produces two transcripts, which result from an alternative splicing of the coding sequence. CTR proteins contain three putative transmembrane regions and an amino-terminal region that is rich in methionine motifs (22). Two or three transmembrane domains were found in the long and short polypeptides of CgCTR2, respectively, which are situated in the middle and at the carboxy terminus of the protein. This configuration is highly similar to the transmembrane domains found in *C. albicans* Ctr1p (16). The shorter transcript was more abundant in RNA samples obtained from fungal cultures and from fungus-infected plants (Fig. 5A and 6A and D), which could suggest different roles of the two peptides. However, both transcripts were fully functional in yeast (Fig. 3), and when overexpressed in *C. gloeosporioides*, the large transcript was predominant (Fig. 6B). Therefore, the functional significance of the alternative splicing of CgCTR2 remains unclear.

Complementation of strain N159 with an intact copy of CgCTR2 fully restored the wild-type phenotype, confirming that the observed phenotypes of the N159 and RNAi strains resulted from reduced CgCTR2 expression. The *M. grisea* CTR2 homolog MgCTR2 also complemented strain N159 although germination was not fully restored, possibly because the MgCTR2 gene was expressed from its own promoter. Exchanging the tightly regulated promoters between these fungi resulted in different expression patterns (14) and therefore
might not be as efficient as expressing the gene from the native promoter.

Dormant spores contain various transcripts, some of which disappear soon after spore germination. These genes might be needed for activation of growth in dormant spores upon receiving the right signals (17). CgCTR2 was highly expressed in resting spores, and the transcript and protein quickly disappeared during pathogenic germination, suggesting that CgCTR2 may be required for the activation of pathogenic germination, which represents the onset of pathogenic development. One explanation for the specific effect of the CgCTR2 silencing on only pathogenic germination would be that copper-requiring enzymes that are involved in activation of the initial stages of pathogenic germination depend on an intracellular copper supply. The high-level expression of CgCTR2 in resting spores indicates that supplying copper to cytoplasmic enzymes does not depend on de novo synthesis of proteins and can take place without any delay at the onset of germination by transport of copper from the vacuole. Indeed, addition of copper to the medium did not overcome the germination defects of Cgctr2 mutants, but it caused a 15% increase in germination of the wild type as well as mutant spores, further demonstrating that copper is necessary for the early stages of germination.

The role of copper in the early stages of fungal pathogenesis is supported by microarray data derived from in planta experiments, in which a number of copper-related genes are found among fungal transcripts that are differentially upregulated (9, 20, 28). We also found a high frequency of expressed sequence tags of several copper-related genes in a cDNA library prepared from pathogenic, germinated spores, including the Ctr3p and Ctr2p copper transporters, the copper chaperone TahA, Cu-transporting P1-type ATPase Crd1p, and a multicopper oxidase (S. Barhoorn and A. Sharon, unpublished data). Deletion of a Colletotrichum lindemuthianum copper-transporting ATPase CLAP1, which is involved in intracellular delivery of copper to copper-requiring enzymes, resulted in mutants that were unable to induce disease symptoms, further demonstrating the importance of proper intracellular copper transport in fungal pathogenesis (18).

*C. gloeosporioides* and *M. grisea* ctr2 mutant strains showed increased sensitivity to hydrogen peroxide, whereas overexpression of CgCTR2 increased resistance to oxidative stress. Due to its ability to adopt both oxidized and reduced states, copper is an important redox cofactor in many copper-dependent enzymes such as polyphenol oxidase, cytochrome c oxidase, and copper/zinc SOD (34). *C. albicans*, yeast, and human ctrl1-null mutants also display increased sensitivity to hydrogen peroxide, which was explained by copper/zinc SOD defects (16, 22). In agreement with this possibility, we showed that low levels of copper (but not iron) reduced sensitivity of CgCTR2 mutants to hydrogen peroxide and reversed it to wild-type levels, whereas the same copper concentrations had no effect on the wild-type sensitivity to hydrogen peroxide (Table 3). These results demonstrate the importance of copper in oxidative stress resistance and the involvement of Cgctr2 in maintaining optimal levels of free copper in the cytoplasm.

Differences were found in the sensitivity of *C. gloeosporioides* and *M. grisea* ctr2 mutants to oxidative stress at different stages of development, with appressorium formation being the most sensitive. The *C. lindemuthianum* clap1 (copper-transporting ATPase) mutant is characterized by beige mycelium and appressoria instead of the normal black pigmentation of these organs (18). Addition of CuSO₄ restored the black pigmentation in clap1 mycelium but not in the appressoria of the mutant, indicating that appressorium melanization might depend on internal copper translocation, which directs copper to the correct cellular compartments (18). In other organisms such as plants or yeasts, many genes are involved in copper uptake, distribution, and sequestration through copper-responsive transcription factors. Once the copper is inside the cell, Cu⁺² chaperones mediate intracellular copper delivery to specific targets such as the mitochondria, vacuole, chloroplast, and the secretory pathway (22, 24). The mechanism of communication between intracellular compartments and cupro-proteins in the cytosol and the plasma membrane is still unknown, but CTR2 is probably an important player in this intracellular copper-distributing apparatus.

The Cgctr2 mutant strains had reduced pathogenicity. The difference from wild type was quantitative and could be compensated for by increasing the number of spores used for plant inoculation. No defects were found in appressorium formation or penetration in pea leaves and onion epidermis assays, and disease symptoms developed on the same time scale as in wild-type-infected leaves. These results suggest that the reduced pathogenicity of the Cgctr2 mutants might be a consequence of reduced levels of pathogenic germination rather than a direct defect in pathogenicity. However, other factors that have not yet been determined could be responsible for this phenotype.

Free sugars are sufficient to activate germination in many fungi. In a classical scenario, dormant spores are activated by, e.g., glucose and then undergo isotropic growth, which can last for several hours, before polarized growth and cell cycle are initiated. Spores of plant-pathogenic fungi germinate in a nutrient-depleted environment and therefore must respond to stimuli that come from their host plant. Because of the lack of an external nutritional source, spores of plant pathogens must rely on internal resources to quickly complete the developmental stages that are necessary for host penetration. Here, we showed that a vacuolar copper transporter that is highly expressed in resting spores (CgCTR2) is necessary for optimal germination. Previous works showed that many fungal copper metabolic genes are induced during pathogenesis, and proper copper translocation by the copper-transporting ATPase CLAP1 is essential for pathogenesis in *C. lindemuthianum* (18). Our results show that copper is needed at the initial stages of pathogenesis in *C. gloeosporioides* and that the putative vacuole copper transporter CgCTR2 is probably involved in this process.

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**REFERENCES**


