Evidence for Transceptor Function of Cellodextrin Transporters in *Neurospora crassa*[*]

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Background: Mechanisms filamentous fungi use to sense cellulose in the environment remain unclear.
Results: *Neurospora crassa* cellodextrin transporters, CDT-1 and CDT-2, are required to induce the expression and secretion of cellulases, using a mechanism distinct from that in *Trichoderma reesei*.
Conclusion: CDT-1 and/or CDT-2 may act as transporting receptors (transceptors).
Significance: CDT-1- and/or CDT-2-mediated cellodextrin signaling may be conserved in industrially useful fungi.

Neurospora crassa colonizes burnt grasslands and metabolizes both cellulose and hemicellulose from plant cell walls. When switched from a favored carbon source to cellulose, *N. crassa* dramatically up-regulates expression and secretion of genes encoding lignocellulolytic enzymes. However, the means by which *N. crassa* and other filamentous fungi sense the presence of cellulose in the environment remains unclear. Previously, we have shown that a *N. crassa* mutant carrying deletions of three β-glucosidase enzymes (Δ3βG) lacks β-glucosidase activity, but efficiently induces cellulase gene expression and cellulolytic activity in the presence of cellobiose as the sole carbon source. These observations indicate that cellobiose, or a modified version of cellobiose, functions as an inducer of lignocellulolytic gene expression and activity in *N. crassa*. Here, we show that in *N. crassa*, two cellodextrin transporters, CDT-1 and CDT-2, contribute to cellulose sensing. A *N. crassa* mutant carrying deletions for both transporters is unable to induce cellulase gene expression in response to crystalline cellulose. Furthermore, a mutant lacking genes encoding both the β-glucosidase enzymes and cellodextrin transporters (Δ3βGΔ2T) does not induce cellulase gene expression in response to cellobiose. Point mutations that severely reduce cellobiose transport by either CDT-1 or CDT-2 when expressed individually do not greatly impact cellobiose induction of cellulase gene expression. These data suggest that the *N. crassa* cellodextrin transporters act as "transceptors" with dual functions - cellodextrin transport and receptor signaling that results in downstream activation of cellulolytic gene expression. Similar mechanisms of transceptor activity likely occur in related ascomycetes used for industrial cellulase production.

Liquid biofuels produced from lignocellulosic biomass are an environmentally clean and renewable source of energy that could displace a significant fraction of the current demand for petroleum (1–4). However, the costs associated with conversion of insoluble polysaccharides in plant cell walls to easily fermentable sugars represent significant barriers to the production of cost-competitive biofuels (4–6). Filamentous fungi have the capacity to secrete large amounts of lignocellulosic enzymes that release fermentable sugars from plant cell walls, and this ability has been exploited by industry to produce cellulases in quantities exceeding 100 g/liter of culture (7).

The best inducers of plant cell wall-degrading enzyme expression by filamentous fungi are insoluble substrates that include cellulose, hemicellulose, or mixtures of plant polymers. Since these naturally inducing substances cannot enter fungal cells, it is generally believed that oligosaccharides released from the polymers and their derivatives function as the actual molecules that trigger enzyme induction (8). Early work in *Trichoderma reesei* using [U-14C]cellobiose confirmed the presence of a cellobiose uptake system (9). This uptake system was specific for β-linked diglucosides, could be inhibited by glucose and uptake was stimulated following treatment with sophorose. Despite characterization of this cellobiose uptake system, no specific transporter or transporters were described to enable the diglucoside uptake. More recently, studies in *T. reesei* identified four different transporters involved in cellulase production in response to a media containing lactose or cellobiose. In the first study, deletion of one transporter (Trire2: 3405) was sufficient to prevent both lactose uptake and cellulase induction in *T. reesei* (10). However a second study using a different *T. reesei* strain found that deletion of the genes encoding either Trire2:77517 or Trire2:79202 showed a decrease in both lactose uptake and cellulase induction (11). While both studies identified different transporters involved in cellulase induction via lactose, the mechanisms of cellulase induction by sophorose (another soluble inducer of cellulases in *T. reesei*) and by crystalline cellulose (Avicel) remained unknown. In a third study using sophorose, cellobiose, or crystalline cellulose as inducers, a dual cellobiose/glucose transporter named Stp1 (Trire2:47710) was shown to be involved in the *T. reesei* carbon catabolite repression response (12). Intriguingly, although dele-
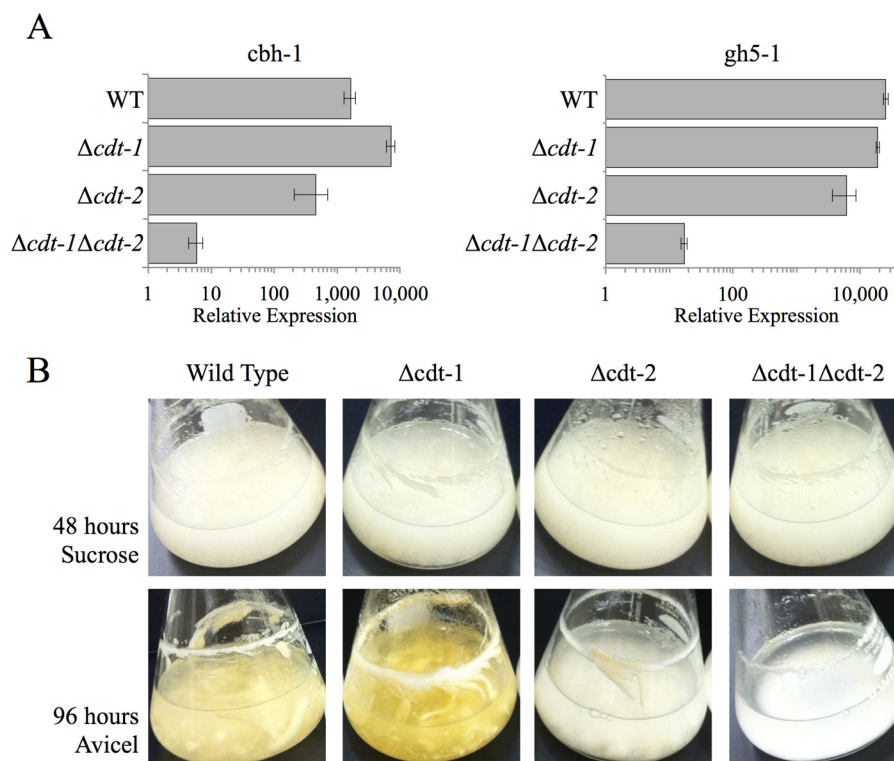
FIGURE 1. **Cellulase gene expression levels in WT and cellodextrin transporter deletion strains after induction with Avicel.** *A*, gene expression of the cellulases *cbh-1* and *gh5-1* after 4 h induction with 1% Avicel in WT, Δ*cdt-1*, Δ*cdt-2*, and Δ*cdt-1*Δ*cdt-2* strains. Gene expression levels of *cbh-1* and *gh5-1* were normalized to 1 when induced with 1% sucrose. Expression levels of actin (NCU04173) were used as an endogenous control in all samples. Each strain was grown in triplicate, and error bars indicate 1 S.D. *B*, growth phenotype of WT, Δ*cdt-1*, Δ*cdt-2*, and Δ*cdt-1*Δ*cdt-2* strains when grown in sucrose or Avicel. Strains were grown for 48 h on 2% sucrose or 96 h on 2% Avicel.

tion of Trire2:3405 abolished lactose uptake (10), the deletion strain still imported sophorose and cellobiose. However, deletion of Trire2:3405 (Crt1) abolished the accompanying induction of cellulases (12). These data suggest that, similar to nutrient sensing in *Saccharomyces cerevisiae* (13, 14), *T. reesei* uses multiple transporters that may function in signal transduction (so called transceptors) to induce cellulase gene expression.

The filamentous fungus *N. crassa*, has been used as a model laboratory organism for nearly 100 years (15). In nature, *N. crassa* is only associated with plant material, primarily killed by exposure to fire (16–19). *N. crassa* is an effective degrader of lignocellulose (20). When grown on crystalline cellulose, *N. crassa* increases the transcription of seven major facilitator superfamily (MFS)[3] sugar transporters (21). Two of these transporters, CDT-1 and CDT-2, were capable of transporting cellodextrins when expressed in *Saccharomyces cerevisiae* (22). In this study, we examine the hypothesis that CDT-1 and CDT-2 function as transporters and also are required for signaling for cellulase induction by crystalline cellulose. We show that the presence of at least one of the transporters CDT-1 or CDT-2 is required for cellulase gene induction and that deletion of both *cdt-1* and *cdt-2* results in a *N. crassa* strain that is unable to sense the presence of crystalline cellulose. Furthermore, by using strains lacking the genes encoding three β-glucosidase enzymes (Δ3βG) (23), we show that additional absence of both

cdt-1 and *cdt-2* results in a strain of *N. crassa* that is unable to sense the presence of cellobiose.

EXPERIMENTAL PROCEDURES

Deletion Strains—Strains obtained from the Fungal Genetics Stock Center (FGSC) (24) include the WT (FGSC 2489), and deletion strains for the two cellodextrin transporters: NCU00801 (FGSC 16575) and NCU08114 (FGSC 17868 and 17869). The Δ3βG deletion strain was described previously (23). Multiple deletion strains were made by performing sequential crosses performed on Westergaard media (25). The genotype of each multiple deletion strain was confirmed using a gene-specific primer and a common primer for the hygromycin phosphotransferase (hph) resistance cassette. The primer for hph was 5′-CGA CAG ACG TCG CGG TGA GTT CAG-3′. Reverse primers were NCU00801: 5′-TAG GGT TGT AGA CAC CTG C-3′; NCU08114: 5′-GAC GAC CAG AAC TAG GTA GG-3′; NCU00130: 5′-TAG TGT ACA AAC CCC AAG C-3′; NCU004952: 5′-AAC ACA CAC ACA CAC ACT GG-3′; and NCU08755: 5′-ACA GTG GAG GTG AGA AAG G-3′.

Shake Flask Studies—Conidia from deletion strains were inoculated at a concentration equal to 10^6 conidia per milliliter in 50 ml of Vogel's salts (26) with 2% w/v sucrose, 2% Avicel PH 101 (Sigma), or 2% cellobiose (Sigma) in a 250 ml Erlenmeyer flask and grown under constant light at 200 rpm. Images were taken at 48, 72, and 96 h.

Transcriptional Studies—Conidia from strains were inoculated at a concentration equal to 10^6 conidia per milliliter in 50

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[3] The abbreviations used are: MFS, major facilitator superfamily; CDT, cellodextrin transporter; hph, hygromycin phosphotransferase.

ml Vogel's salts (26) with 2% w/v sucrose in a 250 ml Erlenmeyer flask and grown under constant light at 200 rpm for 16 h. Biomass was then spun at $1,000 \times g$ for 10 min and washed in Vogel's salts twice to remove any excess sucrose. Biomass was then added to a new flask containing 50 ml of Vogel's salts supplemented with 1% w/v sucrose, 1% w/v Avicel PH 101 (Sigma) or cellobiose (Sigma) at the indicated concentration. Cultures were induced for 4 h under constant light at 200 rpm (23). The culture biomass was then harvested by filtration over a Whatman glass microfiber filter (GF/F) on a Büchner funnel and washed with 50 ml of Vogel's salts. The biomass was flash frozen in liquid nitrogen and stored at $-80\,°C$. Three independent biological replicates (flasks) were evaluated for each time point.

RNA Isolation—Total RNA from frozen samples was isolated using Zirconia/silica beads (0.5 mm diameter; Biospec) and a Mini-Beadbeater-96 (Biospec) with 1 ml TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The total RNA was further purified by digestion with TURBO DNA-free (Ambion) and an RNeasy kit (Qiagen). RNA concentration and integrity was checked by Nanodrop and agarose gel electrophoresis, respectively.

RT-PCR—Quantitative RT-PCR was performed using the EXPRESS One-Step SYBR GreenER Kit (Invitrogen) and the StepOnePlus Real-Time PCR System (Applied Biosystems). Reactions were performed in triplicate with a total reaction volume of 10 μl including 300 nM each forward and reverse primers and 75 ng of template RNA. Data analysis was performed using the StepOne Software (Applied Biosystems) with the Relative Quantitation/Comparative CT ($\Delta\Delta$CT) setting. Data were normalized to the endogenous control actin (NCU04173) with expression on sucrose as the reference sample.

RT-PCR Primers—The primers for actin (NCU04173) were forward 5′-TGA TCT TAC CGA CTA CCT-3′ and reverse 5′-CAG AGC TTC TCC TTG ATG-3′. The primers for *cbh-1* (NCU07340) were forward 5′-ATC TGG GAA GCG AAC AAA G-3′ and reverse 5′-TAG CGG TCG TCG GAA TAG-3′. The primers for *gh5-1* (NCU00762) were forward 5′-GAG TTC ACA TTC CCT GAC A-3′ and reverse 5′-CGA AGC CAA CAC GGA AGA-3′. RT-PCR primers were previously identified and optimized in Tian *et al.* (21) and Dementhon *et al.* (27).

Glucose and Cellobiose Concentration during Growth on Crystalline Cellulose—Conidia from the wild type strain were inoculated in duplicate at a concentration equal to 10^6 conidia per milliliter in 50 ml of Vogel's salts (26) with 2% w/v Avicel PH 101 (Sigma) in a 250 ml Erlenmeyer flask and grown under constant light at 200 rpm for 7 days. Samples were taken daily and spun at $1,000 \times g$ for 10 min to remove biomass and excess Avicel. The concentration of glucose and cellobiose were measured on a DIONEX ICS-3000 HPLC (Dionex Corporation) using a CarboPac PA20 Analytical Column (3×150 mm) and a CarboPac PA20 guard column (3×30 mm) at 30 °C. Following injection of 25 μl of diluted samples, elution was performed with 100 mM KOH (isocratic) at 0.4 ml/min. Sugars were detected using pulsed amperometric detection, Four-Potential Carbohydrate Waveform and Peaks were analyzed using the Chromeleon software package.
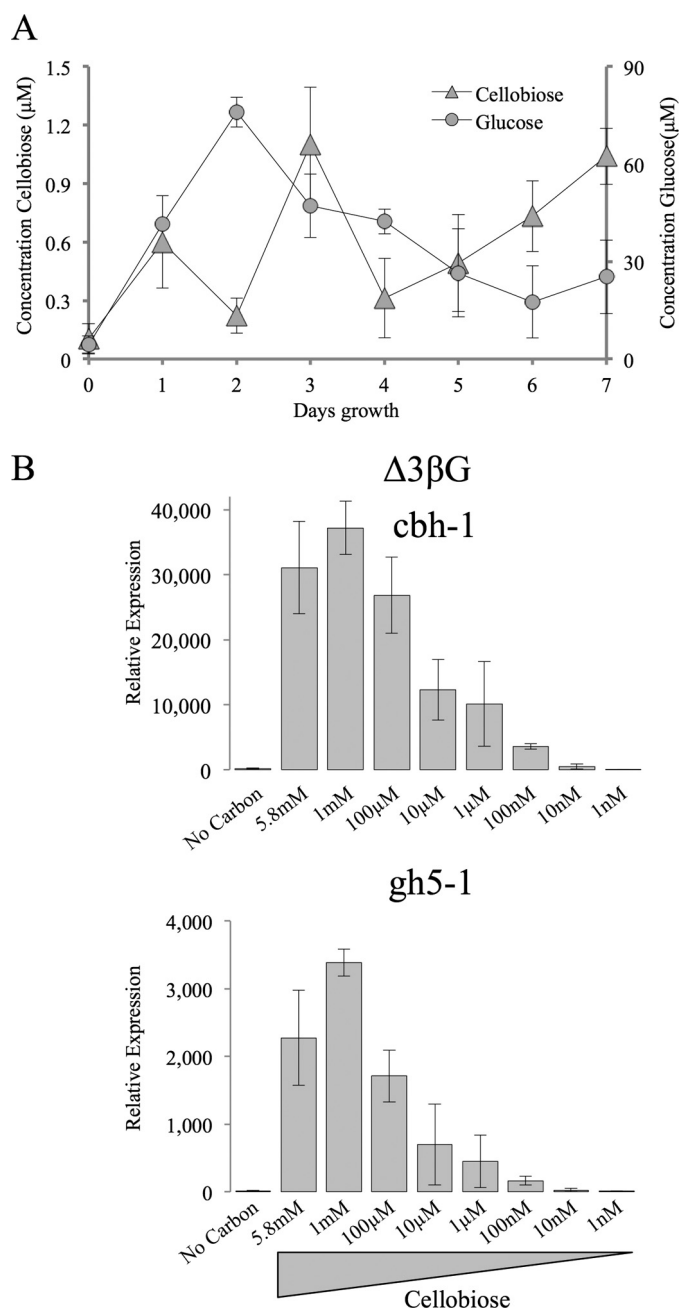

FIGURE 2. **Concentration of glucose and cellobiose in WT cultures and the effect of cellobiose concentration on cellulase gene induction in the $\Delta3\beta$G strain.** *A*, concentration of glucose and cellobiose in WT cultures grown on Avicel. The concentration of glucose and cellobiose were measured every 24 h from cultures growing on 2% Avicel. Samples were run as biological triplicates and error bars indicate 1 S.D. Time point 0 represents the concentration of glucose and cellobiose before inoculation. *B*, gene expression of the cellulases *cbh-1* and *gh5-1* in the $\Delta3\beta$G strain after 4 h induction with varying concentrations of cellobiose. Gene expression levels of *cbh-1* and *gh5-1* were normalized to 1 when induced with 1% sucrose. Expression levels of actin (NCU04173) were used as an endogenous control in all samples. Each strain was grown in triplicate, and error bars indicate 1 S.D.

GFP-tagged Transporters—Template gDNA from *N. crassa* WT strain (FGSC 2489) was extracted according to the method of Lee *et al.* (51). Strains tagged with GFP were created by adapting the tools described in Honda and Selker (28). The 10xGly::GFP::hph::LoxP fragment was subcloned from FJ457011 (28) into pRS426 using XhoI (NEB) and KpnI (NEB).
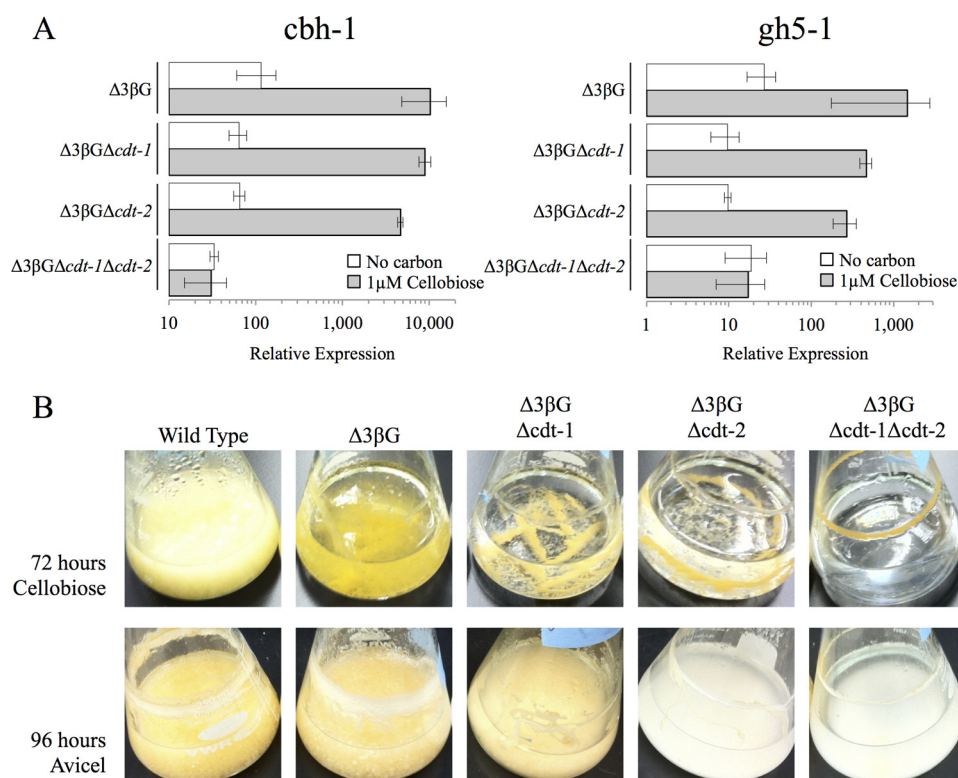
A

B

FIGURE 3. **Cellulase gene expression levels in Δ3βG and cellodextrin transporter deletion strains after induction with cellobiose.** *A*, gene expression of the cellulases *cbh-1* and *gh5-1* after 4 h induction with 1 μM cellobiose or no carbon (starvation) in the Δ3βG, Δ3βGΔcdt-1, Δ3βGΔcdt-2, and Δ3βGΔcdt-1Δcdt-2 strains. Gene expression levels of *cbh-1* and *gh5-1* were normalized to 1 when induced with 1% sucrose. Expression levels of actin (NCU04173) were used as an endogenous control in all samples. Each strain was grown in triplicate, and error bars indicate 1 S.D. *B*, growth phenotype of the Δ3βG, Δ3βGΔcdt-1, Δ3βGΔcdt-2, and Δ3βGΔcdt-1Δcdt-2 strains when grown on cellobiose or Avicel. Strains were grown for 72 h on 2% cellobiose or 96 h on 2% Avicel.

The upstream region was amplified by PCR (~1000 bp upstream and coding region) using primers containing XhoI (NEB) sites and ligated using T4 DNA Ligase (NEB). The downstream region was then amplified by PCR (~1000 bp downstream of the coding region) using primers containing KpnI (NEB) sites and ligated using T4 DNA Ligase (NEB). The final vectors were sequenced using several primers to ensure that the proper sequence was obtained. The primers used to clone the upstream region are as follows NCU08114: 5′-GGG CCC CCC CTC GAG GTC GAC GGT ATC GAT AAG CTT CAA GTT TCG GTA CAC-3′ and 5′-GCC TCC GCC TCC GCC GCC TCC GCC TCG AGA AGC AAC AGA CTT GCC CTC ATG-3′; NCU00801: 5′-GGG CCC CCC CTC GAG GTC GAC GGT ATC GAT ACA TCG TCC CGC CAT CCC GG-3′ and 5′-GCC TCC GCC TCC GCC GCC TCC GCC TCG AGA AGC AAC GAT AGC TTC GGA CA-3′. The primers used to clone the downstream region as as follows: NCU08114: 5′-CGA AGT TAT GGA TCC GAG CTC GGT ACC GTG TCT GTT TGA GAT TG-3′ and 5′-GGG CTG CAG GAA TTC GAT ATC AAG CTG ACG ACC AGA ACT AGG TAG G-3′; NCU00801: 5′-CGA AGT TAT GGA TCC GAG CTC GGT ACC ACA GGC GAC AAG GAA G-3′ and 5′-CTA GTG GAT CCC CCG GGC TGC AGG AAT TCG ATA TCA GCT CGT TGA GCT GTC CC-3′. The vectors were then transformed into the Ku70 deletion strain as described below.

Mutagenesis of CDT-1 and CDT-2 and Strain Construction—Alanine mutants were produced in previously described 2μ yeast expression plasmids (22). These plasmids contained the CDT-1 or CDT-2 open reading frame fused to GFP and the *S. cerevisiae* PGK1 promoter and CYC transcriptional terminator within the pRS426 backbone. The QuikChange (Agilent Technologies) protocol was used to introduce alanine mutations, and results were confirmed by sequencing the plasmids. All plasmids were transformed into the *S. cerevisiae* strain YPH449 (*MATa ura3-52 lys2–801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1*), which contained a previously described (22) plasmid containing the GH1–1 open reading frame and the *S. cerevisiae* PGK1 promoter within the pRS424 backbone.

Determination of Strain Growth Rates—Colonies corresponding to independent transformants of *S. cerevisiae* strain YPH449 containing GH1–1 and a given alanine mutant of CDT-1 or CDT-2 were resuspended in 400 μl of YNP (with ammonium sulfate) plus 2% cellobiose and CSM (-uracil, -leucine) at a starting OD (600 nm) of ~0.2. Growth curves were recorded using a Bioscreen C (Oy Growth Curves Ab Ltd), and growth rates determined from the exponential phase of the curve.

GFP-tagged Point Mutants—GFP-tagged point mutants were created using a QuikChange II XL Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The primers used to create the point mutations are as follows NCU00801 D307A: 5′-ACC GAC GGT GTC GCC AAG GTC TGG TGG-3′ and 5′-CCA CCA GAC CTT GGC GAC ACC GTC GGT-3′; NCU00801 E476A: 5′-GTG TTA TTC CCA CCG CGG CTC TCG AGA CCA-3′ and 5′-GTG GTC TCG
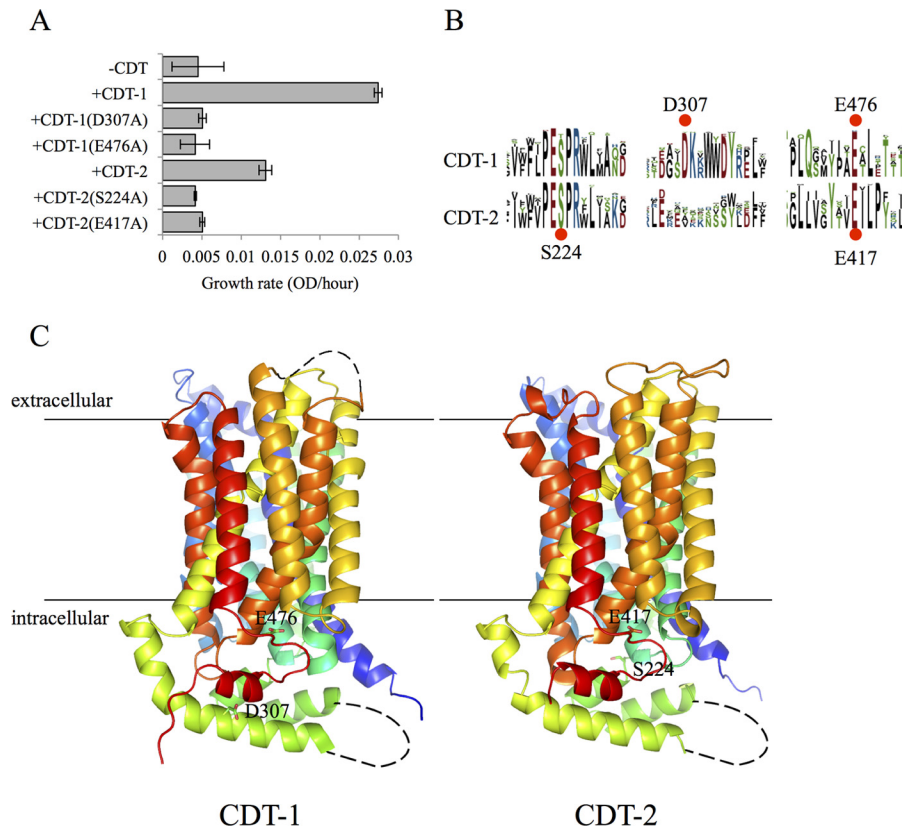
FIGURE 4. **Identification of non-transporting CDT-1 and CDT-2 point mutants.** *A*, cellobiose mediated growth rates of *S. cerevisiae* transformed with CDT-1 or CDT-2 bearing alanine substitutions and GH1–1. Conserved amino acids in CDT-1 and CDT-2 were separately mutated to alanine. A construct containing a mutation at one position was transformed into *S. cerevisiae* alone with the β-glucosidase, GH1–1. These strains were grown with cellobiose as the sole carbon source. The growth rates (increase in the OD at 600 nm per hours) were measured and compared with strains with a wild type CDT and GH1–1 or no transporter and GH1–1. Each sample was performed in duplicate and error bars represent the standard deviation. *B*, sequence logos for CDT-1 and CDT-2 were retrieved from the NCBI BLAST server with an E value cutoff of $1E^{-130}$ and aligned using the MUSCLE algorithm (47). The alignment logos were created with WebLogo (48). The size of the residue letter is proportional to its frequency of appearance at the given position. *C*, homology models of CDT-1 and CDT-2. Homology models of CDT-1 and CDT-2 were built by Modeler v9.12 (49) using a xylose transporter XylE structure; PDB code 4GBZ (50) as the template. The *black lines* indicate the transmembrane region assigned by comparing with XylE structure. Structures were colored in rainbow with N terminus in *blue* and C terminus in *red*. The mutated residues are shown as *sticks*. The figure was prepared with the molecular visualization software PyMol (52).

AGA GCC GCG GTG GGA ATA ACA A-3′; NCU08114 S224A: 5′-GTG GGT TCC CGA GGC TCC CCG TTT CCT 3′ and 5′-AGG AAA CGG GGA GCC TCG GGA ACC CAC-3′; NCU08114 E417A: 5′-CGG CTA CGC CAT CGC AAT CCT CCC TTA CC-3′ and 5′-GGT AAG GGA GGA TTG CGA TGG CGT AGC CG-3′. The final vectors were sequenced using several primers to ensure that the proper sequence was obtained and were then transformed into the *N. crassa* Ku70 deletion strain (29, 30) as described below.

Transformation of N. crassa Strains—1 μg of plasmid DNA was transformed into the Δ*mus-52* strain (FGSC 9719) by electroporation as described (31). The constructs were targeted to the native locus by homologous recombination and integration of the constructs at the native locus in heterokaryotic transformants was confirmed by GFP fluorescence and PCR with primers designed to the region upstream and downstream of the integration cassette. To recover homokaryotic strains, and repair the *mus-52* gene, transformants were crossed with the Δ3βG strain (23) and hygromycin B (Sigma-Aldrich) resistant/GFP-positive/PEST (Glufosinate-ammonium; Sigma-Aldrich) sensitive progenies were screened for the absence of NCU00130, NCU04952, and NCU08755 as described previously (23).

Cellobiose Uptake Assays—Conidia from each strain were inoculated at a concentration equal to 10^6 conidia per milliliter in 3 ml of Vogel's salts with 2% w/v sucrose in a 24 well plate and grown under constant light at 200 rpm for 40 h. Biomass was transferred to 5 ml of Vogel's salts containing no carbon and was incubated at 450 rpm for 5 min. Biomass was again transferred to 3 ml of Vogel's salts containing 2% *w/v* cellobiose (Sigma-Aldrich) and was incubated under constant light at 200 rpm for 24 h to induce transporter expression. Following induction, biomass was washed three times with Vogel's salts containing no carbon to remove any excess cellobiose and transferred to a new 24-well plate with 3 ml of cellobiose uptake assay solution (5 mM MES pH 6.0, 100 mM NaCl, 100 μM cellobiose) and vortexed at 900 rpm for 10 s. 300 μl samples were taken at 2.5, 10, 20, and 30 min intervals. Samples were centrifuged at 14,000 rpm at 4 °C for 10 min. 200 μl of each sample were transferred to an HPLC vial containing 800 μl of 0.1 M NaOH. The concentration of remaining cellobiose was measured on a DIONEX ICS-3000 HPLC (Dionex Corporation) as described above. The remaining biomass was washed with water, dried for 24 h at 95 °C, and weighed with a Sartorius SE2 ultra-micro analytic balance.
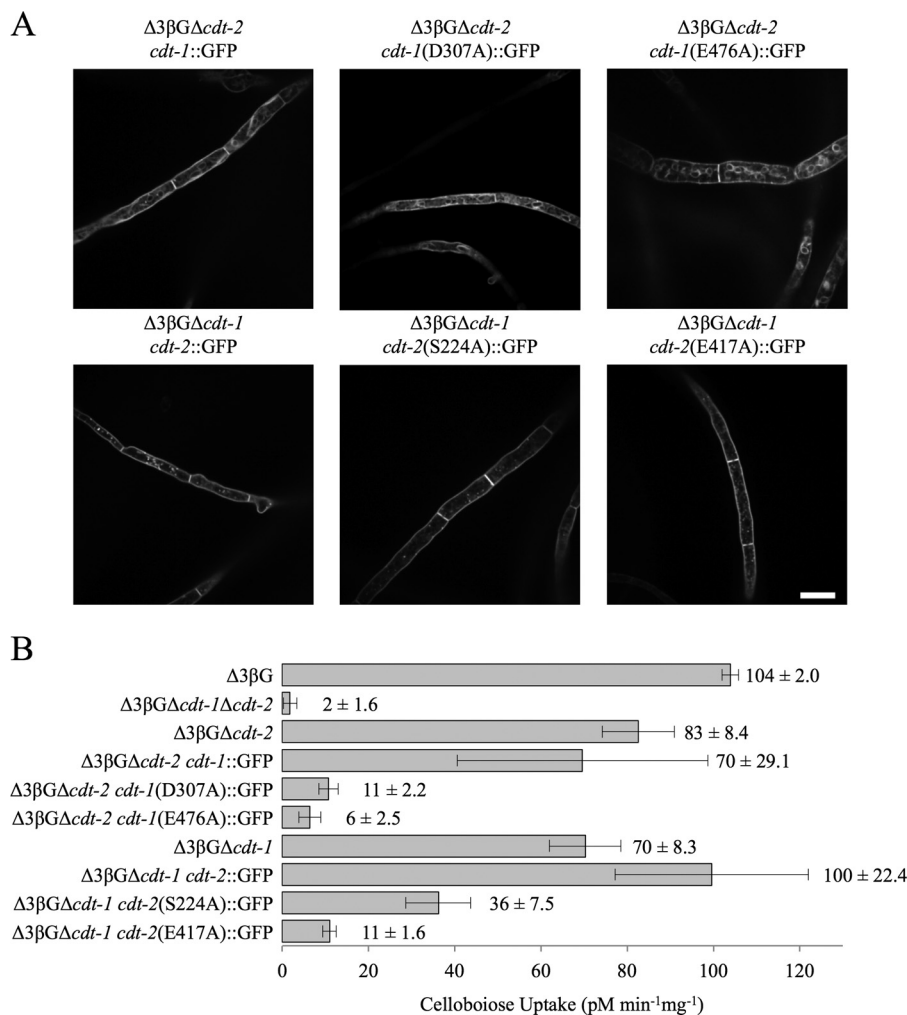
FIGURE 5. **Uptake of cellobiose by *N. crassa* strains expressing untagged and/or GFP-tagged cellodextrin transporters.** *A*, fluorescence microscopy of WT and GFP-tagged point mutants after 24 h induction with 2% cellobiose. Scale bar: 10 μm. *B*, cellobiose uptake in the $\Delta 3\beta G$, $\Delta 3\beta G\Delta cdt$-$1\Delta cdt$-$2$, $\Delta 3\beta G\Delta cdt$-$2$, $\Delta 3\beta G\Delta cdt$-$2$ cdt-1::GFP, $\Delta 3\beta G\Delta cdt$-$2$ cdt-1(D307A)::GFP, $\Delta 3\beta G\Delta cdt$-$2$ cdt-1(E476A)::GFP, $\Delta 3\beta G\Delta cdt$-$1$, $\Delta 3\beta G\Delta cdt$-$1$ cdt-2::GFP, $\Delta 3\beta G\Delta cdt$-$1$ cdt-2(S224A)::GFP, and $\Delta 3\beta G\Delta cdt$-$1$ cdt-2(E417A)::GFP strains after 24 h induction with 2% cellobiose.

Fluorescence Microscopy of GFP-tagged Transporters—Mycelia for microscopy were prepared in the same way as biomass was prepared for cellobiose uptake assays, without the final Vogel's salts washes. Confocal microscopy was performed on a Leica SD6000 microscope equipped with a Yokogawa CSU-X1 spinning disk head and 488-nm lasers controlled by Metamorph software (Molecular Devices). All images were acquired using a 100 × 1.4 NA oil immersion objective.

RESULTS

Induction of Cellulase Gene Transcripts Is Blocked in Cellulose-induced Cultures of N. crassa Lacking Two Cellodextrin Transporter Genes—A previous systems analysis identified several predicted transporters that increase in expression level when *N. crassa* is grown on crystalline cellulose (21). When expressed in *S. cerevisiae*, two of these transporters were capable of efficiently transporting cellooligosaccharides (22). We hypothesized that these up-regulated transporters are involved in sensing of crystalline cellulose and contribute to the induction and secretion of active cellulases. To examine this hypothesis, we initially tested whether the expression of two major cellulase genes (*cbh-1*, NCU07340; *gh5-1*, NCU00762) was induced in strains carrying deletions of the eight previously identified transporters (21) ("Experimental Procedures"). Because strains carrying single deletions were unable to produce a significant defect in induction (Fig. 1*A*), a mutant strain was constructed that lacked both *cdt-1* and *cdt-2*. Unlike the single mutants, this strain (Δcdt-1 Δcdt-2) showed a significant defect in the induction of both *cbh-1* and *gh5-1* following a 4-h transfer of *N. crassa* to 1% w/v Avicel (Fig. 1*A*). In addition to the induction phenotype, we also examined the growth phenotype of strains carrying both single deletions and double deletions of *cdt-1* and/or *cdt-2* on both sucrose and Avicel as compared with wild type (Fig. 1*B*). All strains were able to grow equally well on sucrose. However, a minor growth defect was seen in strain lacking *cdt-2* after 96 h growth on 2% w/v Avicel. A severe growth defect was observed when a strain lacking both *cdt-1* and *cdt-2* was cultivated on Avicel. These data indicate that *cdt-1* and *cdt-2* are involved in both the induction of cellulases as well as the growth of *N. crassa* on crystalline cellulose. Deletion of both genes was sufficient

to prevent *N. crassa* from sensing the presence of crystalline cellulose in its environment.

Deletion of cdt-1 and cdt-2 in a Strain Lacking Genes Encoding Three β-Glucosidase Enzymes (Δ3βG) Results in a Strain Unable to Respond to Cellobiose—Previously, we showed that a *N. crassa* strain lacking genes encoding three β-glucosidase enzymes (two extracellular and one intracellular; Δ3βG) responds to cellobiose in the same way that wild type *N. crassa* responds to crystalline cellulose (23). We therefore hypothesized that a strain lacking the genes encoding the three β-glucosidase enzymes as well as *cdt-1* and *cdt-2* (Δ3βGΔcdt-1Δcdt-2) would be unable to induce cellulases in response to a physiologically relevant concentration of cellobiose. To examine this hypothesis, we first defined the physiologically relevant concentration of cellobiose required for cellulase induction in *N. crassa*.

We first measured the concentration of soluble glucose and cellobiose from cultures growing over 7 days on 2% w/v Avicel (Fig. 2*A*). The glucose concentration reached a maximum of ~70 μM on the second day and proceeded to decrease until day 6 when it reached its minimum concentration of ~20 μM. On the other hand, the cellobiose concentration was much lower, reaching a maximum of about 1 μM over the 7 day time course. In a second experiment, we used the Δ3βG background strain to examine the induction of two major cellulase genes (*cbh-1*, NCU07340; *gh5-1*, NCU00762) in response to decreasing concentrations of cellobiose. We confirmed that 0.2% w/v (5.8 mM) cellobiose produced maximal induction (23), but were also able to detect induction of *cbh-1* and *gh5-1* above that seen by starvation (derepression) with concentrations of cellobiose as low as 10 nM (Fig. 2*B*). Taken together, the results of these two experiments indicate that 1 μM cellobiose should serve as a physiologically relevant concentration for examining cellulase gene induction in strains lacking *cdt-1* and/or *cdt-2* in the Δ3βG background strain.

In a 4-h transfer experiment using 1 μM cellobiose, we found that strains lacking either *cdt-1* or *cdt-2* (in the Δ3βG background strain) showed an induction profile for the two major cellulase genes (*cbh-1*, NCU07340; *gh5-1*, NCU00762) similar to that observed in the Δ3βG background strain alone (Fig. 3*A*). However, when both *cdt-1* and *cdt-2* were deleted (Δ3βGΔcdt-1Δcdt-2), the induction profile for both *cbh-1* and *gh5-1* matched the starvation/derepression profile (Fig. 3*A*). These data imply that this strain is unable to sense the presence of cellobiose. In addition to cellulase gene induction, we examined the growth of strains carrying both the single deletion and double deletions of *cdt-1* and/or *cdt-2* (Fig. 3*B*) in the Δ3βG background strain. The Δ3βG strain grows slowly on cellobiose, likely due to the expression of minor predicted β-glucosidase enzymes (23, 32). Although additional deletion of either *cdt-1* or *cdt-2* did not exacerbate this growth defect, a Δ3βG strain carrying deletions of both *cdt-1* and *cdt-2* (Δ3βGΔcdt-1Δcdt-2) showed almost no growth after 72 h on 2% cellobiose (Fig. 3*B*). Taken together, these results reveal that either *cdt-1* or *cdt-2* are required for the induction of cellulose gene expression when *N. crassa* is exposed to cellobiose as well as for its growth on cellobiose or Avicel.
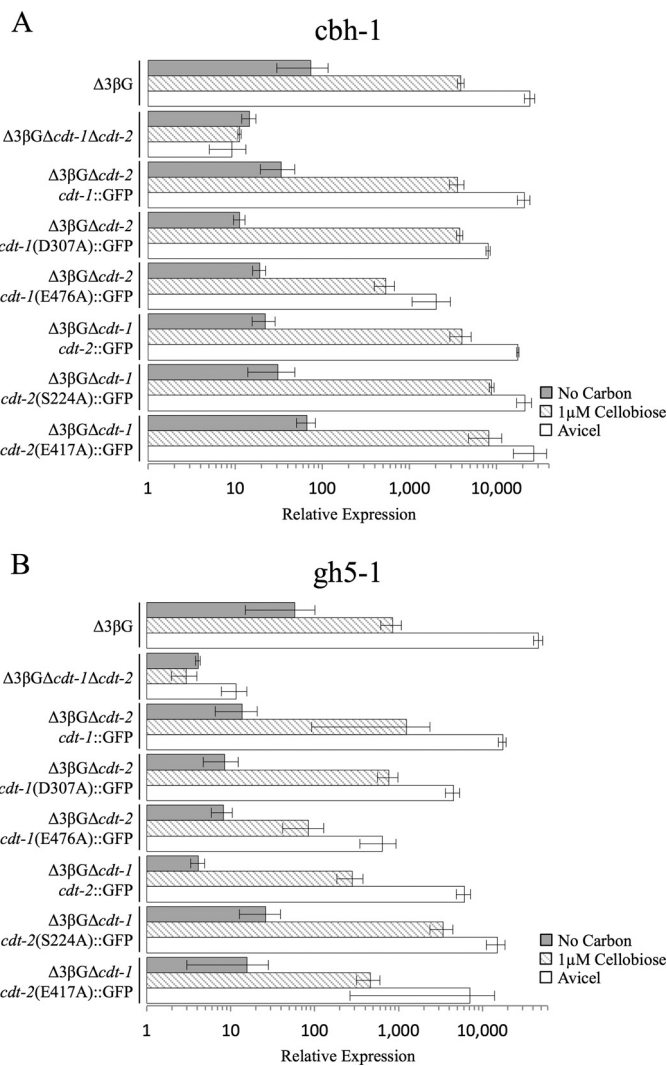
FIGURE 6. **Cellulase gene expression levels in Δ3βG and Δ3βG-cellodextrin transporter mutant strains after induction with cellobiose or Avicel.** Gene expression of the cellulases (*A*) *cbh-1* or (*B*) *gh5-1* after a 4-h induction with 1 μM cellobiose, 1% Avicel or no carbon (starvation) in the Δ3βG, Δ3βGΔcdt-1Δcdt-2, Δ3βGΔcdt-2, Δ3βGΔcdt-2 cdt-1::GFP, Δ3βGΔcdt-2 cdt-1(D307A)::GFP, Δ3βGΔcdt-2 cdt-1(E476A)::GFP, Δ3βGΔcdt-1, Δ3βGΔcdt-1 cdt-2::GFP, Δ3βGΔcdt-1 cdt-2(S224A)::GFP, and Δ3βGΔcdt-1 cdt-2(E417A)::GFP strains. Gene expression levels of *cbh-1* and *gh5-1* were normalized to 1 when induced with 1% sucrose. Expression levels of actin (NCU04173) were used as an endogenous control in all samples. Each strain was grown in triplicate, and error bars indicate 1 S.D.

Point Mutants in CDT-1 and CDT-2 Significantly Decrease Cellobiose Uptake, but Still Allow Cellulase Gene Induction— When heterologously expressed in *S. cerevisiae*, CDT-1 and CDT-2 have been shown to be high-affinity transporters, with CDT-1 acting as a proton symporter and CDT-2 acting as a facilitator (33). To identify functionally important residues within the cellodextrin transporters, CDT-1 and CDT-2, several conserved amino acids were individually mutated to alanine. The functional consequence of each mutation was determined by monitoring the growth of *S. cerevisiae* strains expressing the intracellular β-glucosidase, GH1-1, and mutant CDT-1 or CDT-2 transporters, using cellobiose as a sole carbon source, as described previously (22) (Fig. 4*A*). From these libraries of mutant CDT-1 and CDT-2 (34), several completely abolished growth of *S. cerevisiae* on cellodextrins, implying that
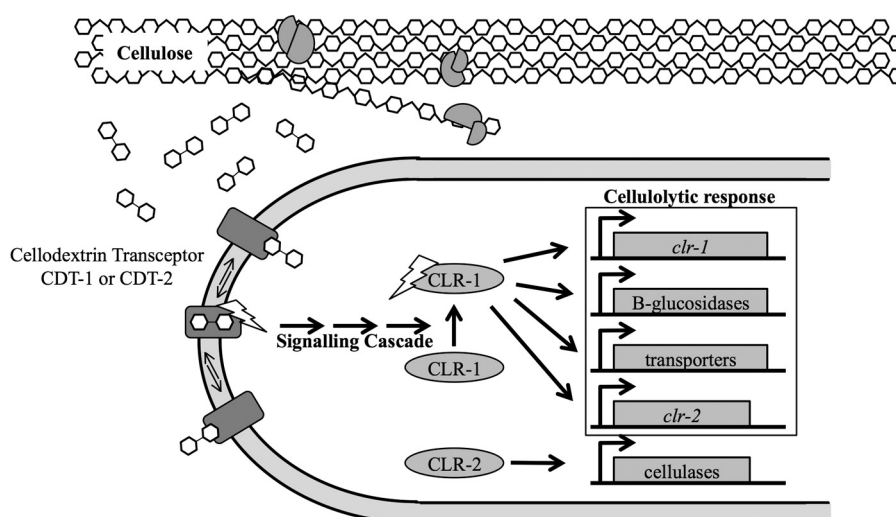
FIGURE 7. **Transporting transceptor model for cellulase induction in *N. crassa*.** Cellobiose is released by cellulases and is recognized by *N. crassa* through either CDT-1 or CDT-2.

these residues were required for cellobiose transport. A comparison of these residues indicates that the sites we chose to mutate are highly conserved (Fig. 4*B*).

The fact that deletion of *cdt-1* and *cdt-2* eliminated nearly all growth of *N. crassa* on cellobiose or Avicel (Fig. 3*B*) allowed us to probe the transport properties of these transporters in their native host. The wild type cellodextrin transporters and point mutants were GFP-tagged and individually transformed into *N. crassa*, replacing the wild type copy of *cdt-1* or *cdt-2* at its resident locus with its GFP-tagged form ("Experimental Procedures"). Only those strains in which mutant CDT-1 or CDT-2 localized properly to the membrane (Fig. 5*A*) were further tested for cellobiose uptake (Fig. 5*B*) and cellulase induction (Fig. 6, *A* and *B*). Comparing cellobiose uptake in the Δ3βG background strain to that in the strain lacking both *cdt-1* and *cdt-2* confirmed that CDT-1 and CDT-2 are the major transporters responsible for cellobiose uptake in *N. crassa* (Fig. 5*B*). Strains carrying a deletion of each transporter individually showed a slight decrease in cellobiose uptake, but as shown earlier (Fig. 3) did not significantly affect the cellulase gene induction by cellobiose. GFP-tagged CDT-1 or CDT-2 transporters also did not significantly alter cellobiose uptake or the ability to induce cellulases in response to cellobiose or Avicel as compared with wild type (Fig. 5, *A* and *B*). Thus, the presence of either *cdt-1* or *cdt-2*, with or without a GFP tag, was sufficient to allow induction of cellulose genes in response to either crystalline cellulose or physiologically relevant concentrations of cellobiose.

As predicted by the *S. cerevisiae* growth assays (Fig. 4*A*), expression of three of the four defective transporters in *N. crassa* resulted in strains in which cellobiose uptake was almost completely abolished (Fig. 5*B*). We hypothesized that if *N. crassa* senses cellobiose intracellularly, abolishing transport should also abolish cellulase gene induction in a similar fashion to what was observed in the strain lacking both transporters (Δ3βGΔ*cdt-1*Δ*cdt-2*) (Fig. 3*A*). We therefore examined the ability of cellobiose or Avicel to induce cellulases in *N. crassa* strains expressing the defective transporters. To our surprise,

three of the four point mutants still promoted induction of *cbh-1* and *gh5-1* in response to either cellobiose or Avicel at levels indistinguishable from wild type (Fig. 6). Particularly noteworthy, the Δ3βGΔ*cdt-1 cdt-2* (E417A)::GFP strain had a 9-fold reduced rate of cellobiose import (Fig. 5*B*), yet had an indistinguishable cellulase induction profile when compared with WT *cdt-2* tagged with GFP (Fig. 6, *strain* Δ3βGΔ*cdt-1 cdt-2*::GFP). The fourth point mutant, Δ3βGΔ*cdt-1 cdt-2* (E476A)::GFP showed a decrease in induction of *cbh-1* and *gh5-1* in response to either cellobiose or Avicel as compared with wild-type with GFP-tagged *cdt-2*, although the induction of *cbh-1* and *gh5-1*was still far above that observed for starvation/derepression, as shown by expression levels in the Δ3βGΔ*cdt-1* Δ*cdt-2* mutant (Fig. 6).

DISCUSSION

In this study, we identified two cellodextrin transporters in the model cellulolytic fungus *N. crassa* (*cdt-1* and *cdt-2*) that are essential for the induction of cellulase gene expression in response to cellulose. Furthermore, in a strain lacking three β-glucosidase genes, one or both transporters are required for sensing the cellulose-derived product cellobiose. Deletion of either *cdt-1* or *cdt-2* does not affect the ability of cellulose or cellobiose to induce cellulase gene expression or activity, indicating that both transporters contribute to cellobiose sensing. However, based on the growth defect for a strain lacking *cdt-2* (22) (Fig. 1*B*), we conclude that *cdt-2* is crucial for sustained growth on cellulose.

Recently, two studies in *T. reesei* identified transporters involved in lactose uptake and strains lacking these lactose transporters no longer induce cellulases in response to lactose. However, because these strains are still able to induce cellulases in response to sophorose (10) or crystalline cellulose (11), these results imply that cellulase signaling via lactose either occurs through an alternative transporter or an alternative mechanism unrelated to the one used during growth on plant cell wall substrates. In a recent study using sophorose, cellobiose or crystalline cellulose as inducers, *T. reesei* was shown to use a "trans-

ceptor" Stp1, a homologue of the receptors Rgt2 and Snf3 in *S. cerevisiae*, to regulate the carbon catabolite repression (12). Nutrient transceptors, transporter-like proteins with a receptor function, have been identified and characterized in several systems including *E. coli* (glucose-6-phosphate sensing (35)), *S. cerevisiae* (nitrogen (36), phosphate (37), and glucose sensing (38)), *Drosophila* (amino acid sensing (39)), *Arabidopsis* (nitrate sensing (40)), as well as in the fungus *Ustilago maydis* (41) and *Candida albicans* (42) for the control of filamentation. Notably, in *T. reesei*, the transceptor Stp1 seems to act independently of the sophorose induction mechanism. By contrast, the *T. reesei* protein Crt1 (Trire2:3405) may act directly as a receptor for cellobiose and sophorose, as its deletion does not impact cellobiose or sophorose import, but abolishes cellulase induction (12). While the studies performed in *T. reesei* utilize single transporter deletion strains to examine transporters involved in cellulase signaling, the data presented in this study utilizes strains containing several deletions as well as site directed mutants to determine how transport of cellobiose *versus* signaling though transceptors mediates the induction of cellulases.

In contrast to *T. reesei*, *N. crassa* uses an alternative mechanism for sensing cellulose. On both crystalline cellulose and cellobiose (the major soluble end product of cellulases) the two transporters CDT-1 and CDT-2 are solely responsible for the ability to of *N. crassa* to sense and grow on cellulose in plant cell walls. Both CDT-1 and CDT-2 seem to act as "transceptors" with the ability for robust cellodextrin transport (21) (Fig. 5*B*) and cellulase gene induction by cellobiose (Fig. 6). While further characterization must be completed to map the structural determinants in CDT-1 and CDT-2 to fully separate nutrient sensing from transport, our studies looking at two point mutations in each transporter have helped to begin to separate transport from signaling and lead to the model presented in Fig. 7. Based on our homology models to the xylose transporter XylE (Fig. 4*C*) homologous point mutants in CDT-1 (E476A) and CDT-2 (E417A) fall within an intracellular unstructured loop region and may block the import of cellobiose by preventing a required conformational change in the protein. The other point mutants in CDT-1 (D307A) and CDT-2 (S224A) fall within an intracellular α-helix and an intracellular transition between an unstructured loop and α-helix, respectively. Their role in blocking the import of cellobiose is less easy to predict due to their further distance from the transmembrane region.

The data presented in this study significantly adds to the model for the induction of cellulases in filamentous fungi, however several questions remain. Although the mutations identified in this study begin to define regions of CDT-1 and CDT-2 required for transport *versus* nutrient signaling, support for a role for CDT-1 and CDT-2 as transceptors will additionally require the identification of the mechanism of intracellular signaling that ultimately results in the activation of the transcription factor CLR-1, the major transcriptional regulator required for induction of cellulase genes, and CLR-2, which also regulates cellulase gene expression in *N. crassa* (Fig. 7) (43, 44). Given the widespread presence of cellodextrin transporters in diverse fungi (22), such signaling networks may also function in a wide range of filamentous fungi of ecological or industrial

interest for biofuels applications, such as the related thermotolerant fungus *Myceliophthora thermophila* (45, 46).

REFERENCES

1. Wyman, C. E. (1999) BIOMASS ETHANOL: Technical Progress, Opportunities, and Commercial Challenges. *Annu. Rev. Energy Environ.* **24,** 189–226
2. Rubin, E. M. (2008) Genomics of cellulosic biofuels. *Nature* **454,** 841–845
3. Carroll, A., and Somerville, C. (2009) Cellulosic biofuels. *Annu. Rev. Plant Biol.* **60,** 165–182
4. Somerville, C. (2007) Biofuels. *Curr. Biol.* **17,** R115–R119
5. Himmel, M. E., and Bayer, E. A. (2009) Lignocellulose conversion to biofuels: current challenges, global perspectives. *Curr. Opin. Biotechnol.* **20,** 316–317
6. Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., and Foust, T. D. (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* **315,** 804–807
7. Cherry, J. R., and Fidantsef, A. L. (2003) Directed evolution of industrial enzymes: an update. *Curr. Opin. Biotechnol.* **14,** 438–443
8. Glass, N. L., Schmoll, M., Cate, J. H., and Coradetti, S. (2013) Plant cell wall deconstruction by ascomycete fungi. *Annu. Rev. Microbiol.* **67,** 477–498
9. Kubicek, C. P., Messner, R., Gruber, F., Mandels, M., and Kubicek-Pranz, E. M. (1993) Triggering of cellulase biosynthesis by cellulose in *Trichoderma reesei*. Involvement of a constitutive, sophorose-inducible, glucose- inhibited β-diglucoside permease. *J. Biol. Chem.* **268,** 19364–19368
10. Ivanova, C., Bååth, J. A., Seiboth, B., and Kubicek, C. P. (2013) Systems Analysis of Lactose Metabolism in *Trichoderma reesei* Identifies a Lactose Permease That Is Essential for Cellulase Induction. *PLoS One* **8,** e62631. 10.1371/journal.pone. 0062631
11. Porciuncula Jde, O., Furukawa, T., Shida, Y., Mori, K., Kuhara, S., Morikawa, Y., and Ogasawara, W. (2013) Identification of Major Facilitator Transporters Involved in Cellulase Production during Lactose Culture of Trichoderma reesei PC-3–7. *Biosci. Biotechnol. Biochem.* **77,** 1014–1022
12. Zhang, W., Kou, Y., Xu, J., Cao, Y., Zhao, G., Shao, J., Wang, H., Wang, Z., Bao, X., Chen, G., and Liu, W. (2013) Two major facilitator superfamily sugar transporters from *Trichoderma reesei* and their roles in induction of cellulase biosynthesis. *J. Biol. Chem.* 10.1074/jbc.M113.505826
13. Thevelein, J. M., and Voordeckers, K. (2009) Functioning and evolutionary significance of nutrient transceptors. *Mol. Biol. Evol.* **26,** 2407–2414
14. Kriel, J., Haesendonckx, S., Rubio-Texeira, M., Van Zeebroeck, G., and Thevelein, J. M. (2011) From transporter to transceptor: signaling from transporters provokes re-evaluation of complex trafficking and regulatory controls: endocytic internalization and intracellular trafficking of nutrient transceptors may, at least in part, be governed by their signaling function. *Bioessays* **33,** 870–879
15. Davis, R. H., and Perkins, D. D. (2002) *Neurospora*: a model of model microbes. *Nat. Rev. Genet.* **3,** 397–403
16. Jacobson, D. J., Powell, A. J., Dettman, J. R., Saenz, G. S., Barton, M. M., Hiltz, M. D., Dvorachek, W. H., Jr., Glass, N. L., Taylor, J. W., and Natvig, D. O. (2004) *Neurospora* in temperate forests of western North America. *Mycologia* **96,** 66–74
17. Jacobson, D. J., Dettman, J. R., Adams, R. I., Boesl, C., Sultana, S., Roenneberg, T., Merrow, M., Duarte, M., Marques, I., Ushakova, A., Carneiro, P., Videira, A., Navarro-Sampedro, L., Olmedo, M., Corrochano, L. M., and Taylor, J. W. (2006) New findings of *Neurospora* in Europe and comparisons of diversity in temperate climates on continental scales. *Mycologia* **98,** 550–559
18. Perkins, D. D., Turner, B. C., and Barry, E. G. (1976) Strains of *Neurospora* collected from nature. *Evolution* **30,** 281–313
19. Turner, B. C., Perkins, D. D., and Fairfield, A. (2001) *Neurospora* from natural populations: A global study. *Fungal Genet. Biol.* **32,** 67–92
20. Znameroski, E. A., and Glass, N. L. (2013) Using a model filamentous fungus to unravel mechanisms of lignocellulose deconstruction. *Biotechnol. Biofuels* **6,** 6
21. Tian, C., Beeson, W. T., Iavarone, A. T., Sun, J., Marletta, M. A., Cate, J. H.,

and Glass, N. L. (2009) Systems analysis of plant cell wall degradation by the model filamentous fungus *Neurospora crassa. Proc. Natl. Acad. Sci. U.S.A.* **106,** 22157–22162

22. Galazka, J. M., Tian, C., Beeson, W. T., Martinez, B., Glass, N. L., and Cate, J. H. (2010) Cellodextrin transport in yeast for improved biofuel production. *Science* **330,** 84–86

23. Znameroski, E. A., Coradetti, S. T., Roche, C. M., Tsai, J. C., Iavarone, A. T., Cate, J. H., and Glass, N. L. (2012) Induction of lignocellulose-degrading enzymes in *Neurospora crassa* by cellodextrins. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 6012–6017

24. McCluskey, K. (2003) The Fungal Genetics Stock Center: from molds to molecules. *Adv. Appl. Microbiol.* **52,** 245–262

25. Westergaard, M., and Mitchell, H. K. (1947) Neurospora V. A synthetic medium favoring sexual reproduction. *Am. J. Bot.* **34,** 573–577

26. Vogel, H. J. (1956) A convenient growth medium for *Neurospora* (medium N). *Microb. Genet. Bullet.* **13,** 42–43

27. Dementhon, K., Iyer, G., and Glass, N. L. (2006) VIB-1 is required for expression of genes necessary for programmed cell death in Neurospora crassa. *Eukaryot Cell* **5,** 2161–2173

28. Honda, S., and Selker, E. U. (2009) Tools for fungal proteomics: multifunctional neurospora vectors for gene replacement, protein expression and protein purification. *Genetics* **182,** 11–23

29. Ninomiya, Y., Suzuki, K., Ishii, C., and Inoue, H. (2004) Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 12248–12253

30. Colot, H. V., Park, G., Turner, G. E., Ringelberg, C., Crew, C. M., Litvinkova, L., Weiss, R. L., Borkovich, K. A., and Dunlap, J. C. (2006) A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 10352–10357

31. Margolin, B. S., Freitag, M., and Selker, E. U. (1997) Improved plasmids for gene targeting at the his-3 locus of *Neurospora crassa* by electroporation. *Fungal Genet. Newslett.* **44,** 34–36

32. Wu, W., Kasuga, T., Nguyen, L., and Fan, Z. (2013) The effects of each β-glucosidase gene deletion on cellulase gene regulation in *Neurospora crassa. A Van Leeuw J. Microb.* published online, 10.1007/s10482-013-9972-7

33. Kim, H., Lee, W. H., Galazka, J. M., Cate, J. H., and Jin, Y. S. (2013) Analysis of cellodextrin transporters from *Neurospora crassa* in *Saccharomyces cerevisiae* for cellobiose fermentation. *Appl. Microbiol. Biotechnol.* 10.1007/s00253-013-5339-2

34. Galazka, J. M. (2011) Cellodextrin Transporters of Neurospora crassa and their Utility in Saccharomyces cerevisiae During a Biofuel Production Process. Ph.D., University of California

35. Schwöppe, C., Winkler, H. H., and Neuhaus, H. E. (2003) Connection of transport and sensing by UhpC, the sensor for external glucose-6-phosphate in *Escherichia coli. Eur. J. Biochem.* **270,** 1450–1457

36. Van Nuland, A., Vandormael, P., Donaton, M., Alenquer, M., Lourenço, A., Quintino, E., Versele, M., and Thevelein, J. M. (2006) Ammonium permease-based sensing mechanism for rapid ammonium activation of the protein kinase A pathway in yeast. *Mol. Microbiol.* **59,** 1485–1505

37. Giots, F., Donaton, M. C., and Thevelein, J. M. (2003) Inorganic phosphate is sensed by specific phosphate carriers and acts in concert with glucose as a nutrient signal for activation of the protein kinase A pathway in the yeast *Saccharomyces cerevisiae. Mol. Microbiol.* **47,** 1163–1181

38. Kraakman, L., Lemaire, K., Ma, P., Teunissen, A. W., Donaton, M. C., Van Dijck, P., Winderickx, J., de Winde, J. H., and Thevelein, J. M. (1999) A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. *Mol. Microbiol.* **32,** 1002–1012

39. Goberdhan, D. C., Meredith, D., Boyd, C. A., and Wilson, C. (2005) PAT-related amino acid transporters regulate growth via a novel mechanism that does not require bulk transport of amino acids. *Development* **132,** 2365–2375

40. Walch-Liu, P., and Forde, B. G. (2008) Nitrate signalling mediated by the NRT1.1 nitrate transporter antagonises L-glutamate-induced changes in root architecture. *Plant J.* **54,** 820–828

41. Smith, D. G., Garcia-Pedrajas, M. D., Gold, S. E., and Perlin, M. H. (2003) Isolation and characterization from pathogenic fungi of genes encoding ammonium permeases and their roles in dimorphism. *Mol. Microbiol.* **50,** 259–275

42. Biswas, K., and Morschhäuser, J. (2005) The Mep2p ammonium permease controls nitrogen starvation-induced filamentous growth in *Candida albicans. Mol. Microbiol.* **56,** 649–669

43. Coradetti, S. T., Craig, J. P., Xiong, Y., Shock, T., Tian, C., and Glass, N. L. (2012) Conserved and essential transcription factors for cellulase gene expression in ascomycete fungi. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 7397–7402

44. Coradetti, S. T., Xiong, Y., and Glass, N. L. (2013) Analysis of a conserved cellulase transcriptional regulator reveals inducer-independent production of cellulolytic enzymes in *Neurospora crassa. Microbiologyopen* **2,** 595–609

45. Rosgaard, L., Pedersen, S., Cherry, J. R., Harris, P., and Meyer, A. S. (2006) Efficiency of new fungal cellulase systems in boosting enzymatic degradation of barley straw lignocellulose. *Biotechnol. Prog.* **22,** 493–498

46. Berka, R. M., Grigoriev, I. V., Otillar, R., Salamov, A., Grimwood, J., Reid, I., Ishmael, N., John, T., Darmond, C., Moisan, M. C., Henrissat, B., Coutinho, P. M., Lombard, V., Natvig, D. O., Lindquist, E., Schmutz, J., Lucas, S., Harris, P., Powlowski, J., Bellemare, A., Taylor, D., Butler, G., de Vries, R. P., Allijn, I. E., van den Brink, J., Ushinsky, S., Storms, R., Powell, A. J., Paulsen, I. T., Elbourne, L. D. H., Baker, S. E., Magnuson, J., Laboissiere, S., Clutterbuck, A. J., Martinez, D., Wogulis, M., de Leon, A. L., Rey, M. W., and Tsang, A. (2011) Comparative genomic analysis of the thermophilic biomass-degrading fungi Myceliophthora thermophila and Thielavia terrestris. *Nat. Biotechnol.* **29,** 922-U222

47. Edgar, R. C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32,** 1792–1797

48. Schneider, T. D., and Stephens, R. M. (1990) Sequence logos: a new way to display consensus sequences. *Nucleic Acids Res.* **18,** 6097–6100

49. Eswar, N., Webb, B., Marti-Renom, M. A., Madhusudhan, M. S., Eramian, D., Shen, M. Y., Pieper, U., and Sali, A. (2007) Comparative protein structure modeling using MODELLER. *Curr. Protoc. Protein Sci.* Chapter 2, Unit 2 9

50. Sun, L., Zeng, X., Yan, C., Sun, X., Gong, X., Rao, Y., and Yan, N. (2012) Crystal structure of a bacterial homologue of glucose transporters GLUT1–4. *Nature* **490,** 361–366

51. Lee, S. B., Milgroom, M. G., and Taylor, J. W. (1988) A rapid, high yield mini-prep method for isolation of total genomic DNA from fungi. *Fungal Genetic Newsletter* **35,** 23

52. DeLano, W. L. (2010) *The PyMOL Molecular Graphics System,* version 1.3r1, Schrödinger, LLC, New York