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Npc1 is involved in sterol trafficking in the filamentous fungus *Fusarium graminearum*

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ABSTRACT

The ortholog of the human gene *NPC1* was identified in the plant pathogenic, filamentous fungus *Fusarium graminearum* by shared amino acid sequence, protein domain structure and cellular localization of the mature fungal protein. The *Fusarium Npc1* gene shares 34% amino acid sequence identity and 51% similarity to the human gene, has similar domain structure and is constitutively expressed, although up-regulated in ungerminated macroconidia and ascospores. GFP-tagged Npc1p localizes to the fungal vacuolar membrane. Cultures derived from a Δ*npc1* mutant strain contain significantly more ergosterol than cultures of the wildtype. Staining with the fluorescent, sterol binding dye filipin, shows that ergosterol accumulates in vacuoles of the Δ*npc1* mutant but not the wildtype strain. The Δ*npc1* mutant has a temperature dependent reduction in growth and greater sensitivity to the ergosterol synthesis inhibiting fungicide tebuconazole compared with the wildtype strain or the mutant complemented with wildtype *Npc1*. The mutant also is significantly reduced in pathogenicity to wheat. Our results are consistent with the interpretation that Npc1p is important for normal transport of ergosterol from the vacuole and is essential for proper membrane function under particular environmental conditions.

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1. Introduction

The process of ergosterol biosynthesis has been exploited for the control of fungal diseases of both plants and animals. Allylamine and azole antifungal compounds each inhibit different enzymatic steps in ergosterol biosynthesis (Ghannoum and Rice, 1999). Despite the importance of these compounds and their action for control of fungal diseases, little is known about regulation of sterol homeostasis and transport within cells of filamentous fungi.

In human, the gene NPC1 appears to be required for proper lipid trafficking from the lysosome (Lloyd-Evans and Platt, 2010). Mutations in the human NPC1 gene result in a fatal autosomal recessive disorder known as Niemann–Pick type C disease (Munkacsi et al., 2009). Niemann–Pick type C disease (NPC) is associated with accumulation of cholesterol and other lipids derived from low-density lipoprotein (LDL) particles in the late endosome. The cholesterol-binding, Npc1 protein (Npc1p) is located in the endosomal membrane and current models suggest that it may result in translocation of cholesterol from the endosome to the endoplasmic reticulum where the sterol may be utilized as a structural component of the cellular membranes (Kwon et al., 2009). It is currently unclear whether cholesterol binding and translocation may be the primary, or merely an ancillary, function of Npc1p or the major deficiency resulting in NPC.

The goal of this study was to determine if a homologous process of sterol trafficking exists in filamentous fungi and to discover the role of a putative fungal NPC1 ortholog in sterol homeostasis of the fungus. Because of the complex domain structure and multifunctional biochemical properties of human NPC1, a microbial model for protein structure and function would be advantageous. Additionally, because of the importance of ergosterol biosynthesis as a target for antifungal therapy of human and plant diseases, further knowledge of ergosterol utilization and movement is warranted.

2. Materials and methods

2.1. Mutant generation

The split marker recombination procedure (Catlett et al., 2003) was used for gene replacement mutation of *Npc*1 (FGSG_09254.3) in *Fusarium graminearum* strain PH-1 (NRRL 31084) (Supplemental Fig. 1). Mycelium was grown 7 days in Complete Medium (CM)

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(Correll et al., 1987) and DNA was extracted according to a published protocol (Pasquali et al., 2004). PCR was performed using primers listed in Table S1. PCR products were purified with QIAquick PCR Purification kit (Qiagen, Valencia, CA). Protoplast preparation and fungal transformation were performed as described previously (Hou et al., 2002). Transformants were cultivated on V8 juice agar (Seong et al., 2009) with 250 μg/ml hygromycin B (Calbiochem, La Jolla, CA). Single spore isolates were preliminarily screened by PCR and two candidate mutants were retained and analyzed by Southern blot as described below. In addition, PCR was used for verification of mutants with primers 9254-N1F and 9254-N2R (Table S1), using a rapid microwave treatment of the fungal samples for DNA extraction (Pasquali et al., 2010). One mutant was chosen for further study and designated PH-1 *Δnpc1*.

To complement the Δ*npc1* mutant, wildtype *Npc1* was amplified from strain PH-1 genomic DNA using primers FGSG_09254FP and FGSG_09254RP (Table S1) that were modified with 5′ phosphate to permit ligation into a dephosphorylated vector. The resulting product was purified using a QIAquick PCR purification kit (Qiagen) and pSM334 (Hou et al., 2002) was completely digested with EcoRV and dephosphorylated using Antarctic phosphatase (New England BioLabs, Ipswich, MA) to prevent re-circularization. The linear vector and *Npc1* fragment were then ligated using T4 DNA ligase (New England BioLabs) at a 3:1 insert vector ratio and 1 μl of the ligation mixture was used to transform One-Shot TOP10 chemically competent cells (Invitrogen, Carlsbad, CA). Ten colonies were picked and subject to plasmid purification using the QIAprep spin miniprep kit (Qiagen). Restriction digests confirmed the presence of the insert in all ten colonies, and one, designated pNPC1COMP was chosen to transform the Δ*npc1* mutant. Protoplasting and transformation was carried out as previously described (Hou et al., 2002) and yielded two transformants that were cultivated on V8 juice agar supplemented with 250 μg/ml geneticin. One transformant was selected for further study and designated PH-1 Δ*npc1::Npc1*.

To generate Npc1p tagged with green fluorescent protein (GFP), a fusion PCR protocol was used (Yang et al., 2004). Primers GFPF and GFPR (Table S1) were used to amplify a 2.4 kb region of pGFP::hph::loxP (GenBank accession: FJ457011) (Honda and Selker, 2009) containing the GFP and hygromycin resistance genes (Supplemental Fig. 2). A 1 kb region of *Npc1* immediately upstream of the predicted stop codon was amplified with primers 09254LF1F and 09254LF2R (Table S1) using PH-1 genomic DNA. A second fragment (0.7 kb) immediately downstream of the predicted stop codon was amplified with primers 09254RF3F and 09254RF4R (Table S1) using PH-1 genomic DNA. All three amplified fragments were then used as the template in a fusion reaction with primers 09254LF1F and 09254RF4R. The expected 4 kb product was purified using a QIAquick gel extraction kit (Qiagen) and 10 μg of the purified fragment was then used to transform PH-1. Transformants were cultivated on V8 juice agar supplemented with 250 μg/ml hygromycin B. One transformant, designated PH-1*Npc1:gfp*, was subject to Southern analysis (Supplemental Fig. 2).

2.2. Southern hybridization/PCR analysis of strains

Npc1 deletion (PH-1 Δ*npc1*), GFP-tagged (PH-1*Npc1:gfp*) and Δ*npc1* complement (PH-1 Δ*npc1::Npc1)* strains were verified by PCR and Southern analysis. Genomic DNA was extracted from mycelium grown for 5 days in CM (room temperature, shaking at 150 rpm) using a modified CTAB protocol (Gale et al., 2011). PCR amplification with primers 9254-1F and 9254-4R verified deletion of FGSG_09254 and replacement with *hph* gene in PH-1 Δ*npc1*, ectopic integration of complementing FGSG_09254 DNA in PH-1 Δ*npc1::Npc1* and GFP tagging of FGSG_09254 in PH-1*Npc1:gfp*. Southern hybridization was performed as per a published protocol (Goswami et al., 2006). For verification of NPC1 deletion and complementation, 2 μg of genomic DNA was digested with *Bst*XI and probed with an internal fragment of the NPC1 gene amplified with primers FGSG_09254_INTF and FGSG_09254_INTR. For verification of the hygromycin resistance (*hph*) gene in these strains, 1.5 μg of DNA was digested with *Bsr*GI and probed with the *hph* gene fragment amplified from pCX62 with primers HYG/F and HYG/R. For confirmation of GFP tagging, 1.5 μg of genomic DNA was digested with *Bgl*II and probed with the eGFP gene fragment amplified from pGFP::hph::loxP with primers GFPcodF and GFPcodR. The *hph* probe and internal NPC1 fragment probe were used to verify presence of *hph* and NPC1, respectively.

2.3. Pathogenicity testing

Wheat plant growth, pathogenicity tests and mycotoxin analysis were as detailed previously (Goswami and Kistler, 2005) but with the following modifications. Wheat plants cv. Norm were fertilized with Nutricote 13-13-13 type 100 two weeks after planting and with 20-20-20 fertilizer 4 weeks after planting. Inoculum was prepared by growing cultures in 100 ml CMC for 4–5 days at 25 °C, 12 h fluorescent light, with shaking at 150 rpm. Conidia were collected by filtering cultures through one layer of Miracloth and centrifuging the filtrate for 10 min at 2500*g*. Supernatant was discarded and pelleted conidia washed twice with sterile water (centrifuge 10 min, 2500*g*). The concentration of conidia was adjusted to 10^6 conidia per ml water. To aid in adhesion to the wheat spikelet, Triton X-100 was added to a final concentration of 0.1% (v/v). Awns were removed from the central spikelet of wheat heads at anthesis, and the central spikelet inoculated with 10^4 conidia using a micropipette. Inoculated plants were then placed in a dew chamber for 24 h. The door to the chamber was then opened for a half hour, closed and dew applied for another 24 h. Inoculated plants were then transferred to a growth chamber (18 °C, 16 h day, 16 °C night measured at head height) for 14 days. For each strain, ten wheat heads were inoculated per replication. Each strain had four replicates for a total of 40 heads per strain. Head blight symptoms were rated 14 days post inoculation (dpi) by counting the number of symptomatic spikelets per inoculated head (Proctor et al., 1995). A total of 10 spikelets were evaluated per head, four spikelets below the inoculated central spikelet, the inoculated spikelet, and five spikelets above. Following disease evaluation, the directly inoculated central spikelets were processed for mycotoxin analysis. The inoculated spikelet was removed from the head, weighed and placed in a one dram glass vial with a screw cap. Trichothecene extraction and GC/MS analysis were as described previously (Goswami and Kistler, 2005).

2.4. Growth in culture

Cultures from long-term storage, frozen at −80 °C in 50% glycerol, were grown at room temperature on minimal medium (MM) (Correll et al., 1987) for 5 days. From the growing colony edges, 5 mm plugs were punched using a cork borer, removed and a single agar plug placed mycelial side down in the center of a fresh 100 mm plate containing the same agar medium. Plates were marked with three lines to obtain measurements of colony diameter. Inoculated plates were incubated at 25 °C for 4 days or at 32 °C for 7 days. Measurements of fungal growth were taken each dpi along the three marked lines.

2.5. Microscopy

Fluorescence and differential interference contrast (DIC) microscopy were performed with a Nikon Eclipse E800 hyperspectral microscope except for 7-amino-4-chloromethyl coumarin

(CMAC) stained samples that were examined with a Nikon Eclipse 90i microscope. Filipin stained samples were prepared using a previously described protocol for fixing and staining filamentous fungi (Momany, 2001). Briefly, complete medium inoculated with 10^6 conidia was poured into a Petri dish containing a coverslip and incubated for either 8 or 12 h at 25 °C. Coverslips supporting the fungus were then removed and fixed for 30 min in 3.7% formaldehyde and rinsed with ddH_2O. A 5 mg/ml stock solution of filipin (Sigma) dissolved in DMSO was diluted to 25 μg/ml and used to stain the fixed samples for 10 min. Samples were then rinsed with ddH_2O, mounted on a microscope slide and sealed with nail varnish.

Samples expressing GFP were prepared by streaking conidia onto a thin layer of solid minimal medium mounted on a microscope slide using a sterile cotton bud. Samples were then placed in a humid chamber and incubated for the appropriate time at 25 °C. A coverslip was then placed over the area to be examined and a small drop of emersion oil added. For CMAC (Invitrogen, Carlsbad, CA) staining of GFP expressing samples, 10^7 conidia were added to 50 ml minimal medium in a conical flask and incubated for 24 h at 25 °C with constant shaking (150 rpm). Mycelia were then transferred to fresh MM containing 10 μM CMAC and incubated for a further 30 min at 25 °C. A final incubation of 30 min at 25 °C in fresh minimal medium was performed and samples prepared as a wet mounts for microscopic observation.

2.6. Ergosterol measurement

Fungal cultures were grown in CM for 7 days. Filtered, freeze-dried mycelium was then used to determine ergosterol content of three independent cultures each of PH-1 and the FGSG_09254 deletion strain. Measurements were repeated once and mean values compared by a two-tailed *T*-test. Ergosterol content was also performed on infected wheat plants. Three spikes infected either with PH-1 or with the FGSG_09254 deletion were collected 14 dpi. Spikes were dried and ergosterol was measured as previously reported (Dong et al., 2006).

3. Results

3.1. FGSG_09254 is the likely ortholog of human NPC1

A BLASTP comparison of the amino acid sequence of human gene NPC1 (GenBank Accession number AAK25791.1) with proteins predicted for the fungus *F. graminearum* shows highest similarity to the coding sequence of gene FGSG_09254.3. This predicted 1273 amino acid protein has 34% amino acid identity (442/1279) and 52 % similarity (667/1279) to the 1279 amino acid human protein. Moreover, like the human protein, FGSG_09254 encodes a sterol sensing domain (Kuwabara and Labouesse, 2002) consisting of five transmembrane motifs as predicted by the MIPS FGDB automated annotation pipeline (Güldener et al., 2006). Overall, 13 transmembrane motifs are predicted for FGSG_09254.3 using the Hidden Markov Model program TMHMM (Krogh et al., 2001). Amino acid alignment of FGSG_09254.3 with human NPC1 using CLUSTAL 2.0.12 (Chenna et al., 2003) reveals a strong correspondence between the 13 transmembrane domains of NPC1 and the 13 domains predicted using TMHMM for the *Fusarium* protein (Supplemental Fig. 3).

Further search of fungal genome sequences indicates that in nearly all examined ascomycetes (e.g. *Neurospora crassa, Aspergillus nidulans, Fusarium oxysporum, Saccharomyces cerevisiae),* basidiomycetes (*Ustilago maydis*, *Coprinus cinereus, Postia placenta)* and lower fungi (*Rhizopus oryzae*, *Mucor circinelloides, Phycomyces blakesleeanus),* each has a single predicted gene with similar high sequence similarity to the human gene. Alignments generally are around 1200 amino acids (Supplemental Fig. 4). Only the reduced fungal genomes of *Schizosaccharomycetes pombe* and the microsporidia *Encephalitozoon cuniculi, Enterocytozoon bieneusi* and *Nosema ceranae* seem to lack a clear NPC1 homolog. Based on its conservation in most true fungi and shared structural elements with human NPC1, we hereby call FGSG_09254.3, the *F. graminearum* gene Npc1.

Previously published microarray data for *F. graminearum* indicates *Npc*1 is constitutively expressed during growth in culture or in plants (Güldener et al., 2006; Seong et al., 2009). Expression is elevated in ungerminated conidia and especially in ungerminated ascospores (Pasquali et al. unpublished).

3.2. Npc1:GFP localizes to the vacuolar membrane

Intracellular localization of *F. graminearum* Npc1p was examined using a *Npc1:gfp* fusion. A gene replacement construct was designed to substitute the native *Npc1* allele with one tagged at the C-terminus with GFP. A hinge region of ten glycine residues (10XGly) in-frame between the C-terminus of Npc1p and the N-terminus of the GFP was included to help maintain functionality of the fusion protein. The transformant PH-1*Npc1:gfp* was confirmed by PCR and Southern analysis to have the GFP-tagged gene placed under control of the native promoter (Supplemental Fig. 2).

Fluorescence and DIC microscopy of PH-1*Npc1:gfp* revealed the GFP-tagged protein localized to the vacuolar membrane (Fig. 1). Localization was similar to a GFP-tagged vacuolar membrane protein, Vam3p, in *Aspergillus oryzae*. Fluorescence was observed primarily in nascent conidia and hyphae of older cultures (24 h) where vacuoles were most prevalent (Shoji et al., 2006). This observation was consistent with expression levels of *Npc1* during a macroconidium germination time course in *F. graminearum*

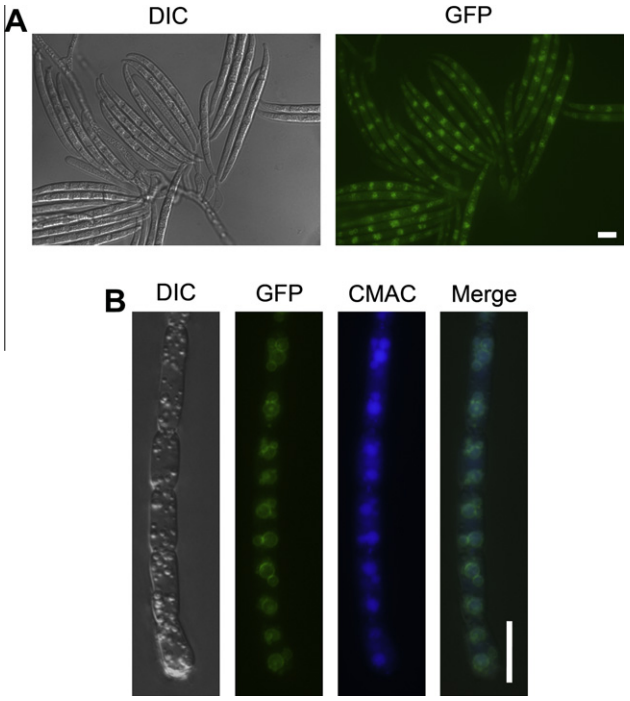

Fig. 1. Npc1:GFP localizes to the vacuolar membrane. (a) PH-1*Npc1:gfp* grown for 48 h. DIC and fluorescence microscopy revealed vacuoles at the sites of GFP localization in nascent conidia. (b) CMAC was used to stain vacuoles in PH-1*Npc1:gfp* grown for 24 h. Npc1:GFP surrounded stained vacuoles in a merged DIC/GFP/CMAC image confirming its localization to the vacuolar membrane. Scale bars, 10 μM.

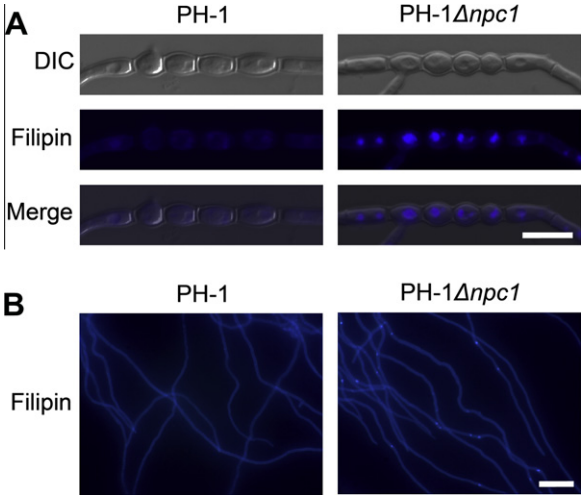

Fig. 2. PH-1Δ*npc1* accumulates ergosterol in the vacuole. Filipin staining of cells grown for 8 h (A) and 12 h (B) revealed an accumulation of ergosterol in vacuoles of the conidium (A) and hyphae (B) in the Δ*npc1* mutant. Vacuolar ergosterol accumulations were absent in the wildtype PH-1. Scale bars, 10 μM (A) and 50 μM (B).

where the highest expression levels were recorded after 24 h growth (Seong et al., 2008). To provide further evidence of the location of Npc1:GFP, the vacuole-specific fluorescent dye, 7-amino-4-chloromethyl coumarin (CMAC), was applied to mycelia of PH-1*Npc1:gfp*. Merging DIC and fluorescence images confirmed the localization of Npc1:GFP to the vacuolar membrane (Fig. 1B). The results are consistent with yeast where the homolog Ncr1p also localizes to the vacuolar membrane (Berger et al., 2007).

3.3. Δnpc1 mutants are altered in ergosterol accumulation

Mutations in human NPC1 prevent endocytosed cholesterol from exiting lysosomes. The resulting accumulation of lipids in this compartment gives rise to NPC disease. The cholesterol binding dye filipin was used to investigate a role for *F. graminearum Npc1* in lipid trafficking. Filipin has previously been shown to reliably detect ergosterol (the fungal equivalent of cholesterol) in dimorphic fungi (Munkacsi et al., 2009), yeasts, and filamentous fungi (Martin and Konopka, 2004; Van Leeuwen et al., 2008).

The localization of ergosterol in an Δ*npc1* mutant (PH-1 Δ*npc1)* was strikingly different than that observed in the wildtype strain PH-1 (Fig. 2). Staining in the wildtype was diffuse with only faint fluorescence at sites of septation, a region of ergosterol localization reported in *Candida albicans* (Martin and Konopka, 2004) and *A. nidulans* (Pearson et al., 2004). In contrast, the Δ*npc1* mutant displayed bright punctate fluorescence, predominantly in newly germinated conidia and throughout older hyphae. Overlaying fluorescence and DIC images revealed ergosterol in the Δ*npc1* mutant was localized to vacuoles. Vacuoles were also present in the wildtype but did not contain appreciable amounts of the lipid (Fig. 2).

To compare ergosterol levels in the Δ*npc1* mutant and wildtype stains, cultures were grown in complete medium and resulting mycelia analyzed for ergosterol content using gas chromatography/mass spectrometry. The mutant mycelium had >30% more ergosterol content (7.9 mg/g dry mycelium) than wildtype (6.0 mg/g), a significant difference ($P = 0.01$). Ergosterol concentrations in plants infected with mutant (416 μg/g dry tissue) or wildtype (333 μg/g) followed the same trend but mean levels were not significantly different ($P = 0.10$).

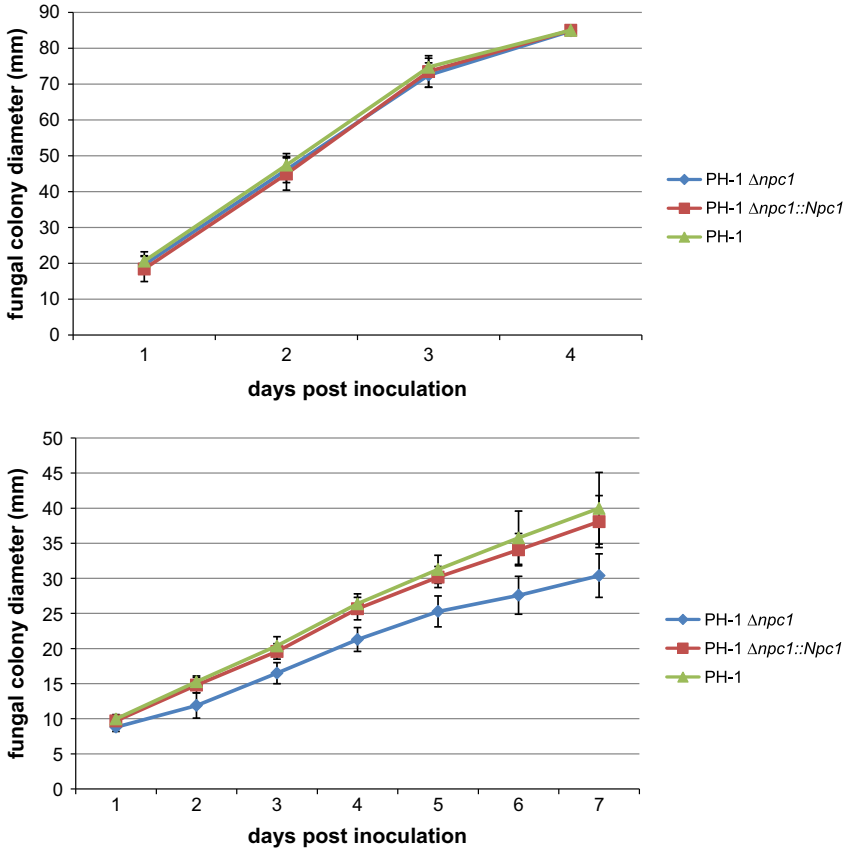

Fig. 3. The growth of wildtype strain PH-1, PH-1 Δ*npc1* and the PH-1 Δ*npc1::Npc1* complement on Petri plates with minimal medium at 25 °C (above) or 32 °C (below).

3.4. Δnpc1 mutants grow slower than wildtype at elevated temperature and are more sensitive to tebuconazole

At 25 °C, the wildtype strain PH-1, the Δ*npc1* mutant PH-1 Δ*npc1* and an Δ*npc1* mutant complement PH-1 Δ*npc1::Npc1* have similar growth kinetics on minimal medium (Fig. 3). However, at 32 °C the growth of the Δ*npc1* mutant is significantly retarded throughout the growth assay compared to the wildtype. This growth defect is reversed by adding back the wildtype *Npc1* gene in the complement. Asexual sporulation or macroconidium germination of the Δ*npc1* mutant did not appear to be appreciably different than wildtype at either temperature (data not shown).

Since deletion of *Npc1* appears to affect trafficking and accumulation of ergosterol, we treated cultures with the azole fungicide tebuconazole, which inhibits ergosterol biosynthesis in fungi, to determine if the mutant strain was more sensitive than wildtype to inhibition. Growth inhibition was tested at the tebuconazole EC_{50} concentration, empirically determined for wildtype strain PH-1 to be 0.1 μg/ml under the conditions tested (data not shown). Tebuconazole at this concentration was slightly more inhibitory to the Δ*npc1* mutant than the wildtype or the *Npc1* complement strain (Fig. 4). Addition of external 0.001% ergosterol did not relieve inhibition of the mutant by tebuconazole (data not shown).

3.5. Δnpc1 mutants cause less disease on a susceptible wheat variety

Mutations affecting expression of genes for enzymes of the isoprenoid biosynthetic pathway leading to ergosterol synthesis previously have been shown to reduce the pathogenicity of these mutants to wheat (Seong et al., 2005, 2009). Wheat pathogenicity tests thus were conducted on the Δ*npc1* mutant to determine if alteration of ergosterol trafficking also reduced the pathogenicity of the fungus. The Δ*npc1* mutant was significantly reduced in the ability to spread within wheat spikelets (mean and std. dev. symptomatic spikelets = 7.2 ± 1.9) compared to wildtype PH-1 (8.7 ± 1.1; $P = 8.71$ e-5) or the mutant complemented with *Npc1* (8.1 ± 1.6; $P = 0.02$). The mutant thus was reduced a small yet significant amount in the ability to cause disease yet was otherwise similar in disease phenotype (Supplemental Fig. 5). Wheat infected with any of the three strains accumulated similar levels of deoxynivalenol toxin; mean toxin concentrations accumulating in inoculated spikelets (PH-1 = 528 ± 148 ppm; Δ*npc1* = 603 ± 203 ppm; Δ*npc1*/Npc1 = 558 ± 149 ppm) were not significantly different.

4. Discussion

Genes likely orthologous to the human *Npc1* gene have been found in *F. graminearum* and other filamentous fungi. The human gene and fungal gene from *F. graminearum* encode proteins of similar size with 52% amino acid sequence similarity across the entire protein. Conservation of domain structure, similar subcellular localization and shared function in sterol trafficking all indicate that the human and *F. graminearum* genes are orthologous proteins shared between the animal and fungal kingdoms.

Individuals with NPC may have point mutations in the NPC1 gene that encode non-synonymous amino acid substitutions at numerous dispersed loci (http://npc.fzk.de/). Because the mutations are associated with the NPC disease phenotype, it is reasonable to assume that many of them are at functionally significant amino acids. If this is the case, then those amino acid positions should be more likely to be conserved over evolutionary time. Comparing the human and *Fusarium* Npc1 sequences, a total of 163 unambiguously aligned amino acid positions contained non-

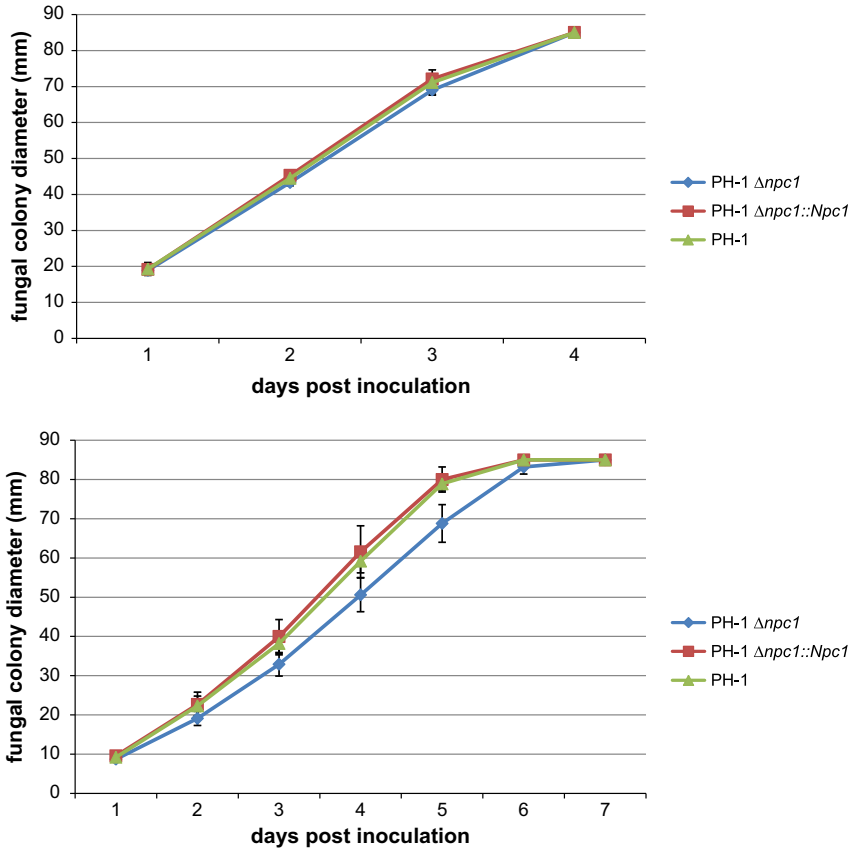

Fig. 4. The growth of wildtype strain PH-1, PH-1 Δ*npc1* and the PH-1 Δ*npc1::Npc1* complement on Petri plates with minimal medium (above) or minimal medium containing 0.1 μg/ml tebuconazole (below).

synonymous amino acids in individuals with NPC disease (Supplemental Fig. 3). While overall amino acid sequence identity between the proteins is 31%, 78 of the 163 positions (48%) are identical between human and *Fusarium*. These positions are significantly more conserved than would be expected merely by chance ($p = 0.0002$). We interpret this finding as evidence for evolutionary constrain at these positions due to shared functional significance.

One functional domain that has diverged between the human and fungal NPC1 proteins is the sterol binding site, likely due to the differences in the dominant sterols in each organism, cholesterol or ergosterol, respectively. The amino acids at the amino terminal domain (NTD) of the human NPC1p determined to be involved in cholesterol binding have the sequence RYN**C**[X_n]T**L**[X_n]NL**FC**[X_n]DN[X_n]**PF**T**I** (Kwon et al., 2009). The corresponding region in *F. graminearum*, contains the sequence LP**C**[X_n]**L**[X_n]**FC**[X_n]GS**PF**Q**I** that, while diverged significantly from the human NPC1p, is highly conserved among fungi and in fact is identical for all examined fungal *Npc1* proteins (Supplemental Figs. 3 and 4). Likewise, the portion of the NTD of human NPC1p responsible for sterol transit to liposomes in an experimental system is conserved among mammals (Kwon et al., 2009) but has little or no amino acid sequence similarity with the *F. graminearum* Npc1p. Nevertheless, this region contains the sequence IGGGAK that is found in all fungal Npc1p. These results suggest a conserved function for these amino acid positions among the fungi that may similarly function by binding ergosterol or other fungal sterols.

Perhaps the most striking phenotype of the Δ*npc1* mutant is the accumulation of sterol in vacuoles (Fig. 2), likely accounting for the elevated levels of ergosterol in the mycelium as a whole. However, increased sensitivity of the mutant to tebuconazole suggests that levels of ergosterol functioning in membranes may actually be reduced due to improper targeting of sterol. Presumably this defect also accounts for the temperature dependent growth reduction and reduced pathogenicity of the mutant.

Taken together, our results demonstrate that the *F. graminearum* Npc1p has many structural and functional similarities to the othologous human protein. Additionally, unlike in *S. cerevisiae* where *NPC1* deletion mutants have no detectable altered phenotype (Berger et al., 2007), mutations in the *F. graminearum Npc1* coding sequence result in altered subcellular- and macroscopic growth phenotypes that reflect the function of the native protein. As such, the gene in *F. graminearum* and perhaps other filamentous fungi may be more useful than yeast as a model for dissecting the multiple functional domains of the human protein. Given the importance of sterol inhibiting fungicides in controlling fungal infections in both plants and animals, further study of ergosterol cellular trafficking mediated by *Npc1* in filamentous fungi also is warranted.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2011.03.001.

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