The EMBO Journal Vol. 22 No. 2 pp. 225–235, 2003

Trehalose synthesis and metabolism are required at different stages of plant infection by *Magnaporthe grisea*

Andrew J.Foster[1], Joanna M.Jenkinson and Nicholas J.Talbot[2]

School of Biological Sciences, University of Exeter, Washington Singer Laboratories, Perry Road, Exeter EX4 4QG, UK

[1]Present address: The Sainsbury Laboratory, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

[2]Corresponding author
e-mail: N.J.Talbot@exeter.ac.uk

The relationship of trehalose metabolism to fungal virulence was explored in the rice blast fungus *Magnaporthe grisea*. To determine the role of trehalose synthesis in pathogenesis, we identified and deleted *TPS1*, encoding trehalose-6-phosphate synthase. A *Δtps1* mutant failed to synthesize trehalose, sporulated poorly and was greatly attenuated in pathogenicity. Appressoria produced by *Δtps1* did not develop full turgor or elaborate penetration hyphae efficiently. To determine the role of subsequent trehalose breakdown, we deleted *NTH1*, which encodes a neutral trehalase. Nth1 mutants infected plants normally, but showed attenuated pathogenicity due to a decreased ability to colonize plant tissue. A second trehalase was also identified, required both for growth on trehalose and mobilization of intracellular trehalose during infection-related development. *TRE1* encodes a cell wall-localized enzyme with characteristics of both neutral and acidic trehalases, but is dispensable for pathogenicity. Our results indicate that trehalose synthesis, but not its subsequent breakdown, is required for primary plant infection by *M.grisea*, while trehalose degradation is important for efficient development of the fungus in plant tissue following initial infection.

Keywords: *Magnaporthe grisea*/metabolism/trehalase/virulence

Introduction

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a non-reducing disaccharide that is commonly found as a storage carbohydrate in eukaryotic cells (Arguelles, 2000) and implicated in the response to various environmental stresses (Thevelein, 1984, 1996). In *Saccharomyces cerevisiae*, trehalose has been shown to be required by cells to contend with diverse stresses such as heat shock, starvation and desiccation (De Virgilio *et al*., 1994). Trehalose is also known to protect enzymes from thermal stress *in vitro*, providing a potential means for cells to survive during adverse conditions (Hottiger *et al*., 1994).

Investigations to determine the biological function of trehalose in eukaryotes have been limited predominantly to the study of free-living species. Consequently, little is known about trehalose metabolism in pathogenic microorganisms. Many virulence-associated functions, including infection-related development and colonization of host tissue, require pathogens to respond to rapid changes in external environment and to mobilize storage carbohydrates. In this study, we set out to determine the role of trehalose metabolism in a plant pathogenic fungus, *Magnaporthe grisea*. This pathogen is the causal agent of rice blast disease, the most serious disease of cultivated rice (Talbot and Foster, 2001).

Trehalose metabolism in eukaryotic cells has been studied in most detail in *S.cerevisiae*. Here, trehalose is synthesized by a multienzyme complex containing trehalose-6-phosphate synthase (T6PS) encoded by *TPS1*, a trehalose-6-phosphatase encoded by *TPS2*, and two regulatory subunits encoded by the *TSL1* and *TPS3* genes (Vuorio *et al*., 1993; Bell *et al*., 1998). Trehalose-6-phosphate (T6P) is synthesized using UDP-glucose and glucose-6-phosphate as substrates, and then directly converted to trehalose. Three distinct trehalases are present in *S.cerevisiae*. Acidic trehalase, encoded by *ATH1*, is required for utilization of trehalose as a carbon source and is a vacuolar enzyme with optimum activity at low pH (Londesborough and Varimo, 1984; Mittenbühler and Holzer, 1988). Mobilization of intracellular trehalose is catalysed by cytoplasmic neutral trehalase, encoded by *NTH1* and *NTH2* (Nwaka and Holzer, 1998). In filamentous fungi such as *Aspergillus nidulans*, trehalose synthesis involves T6PS encoded by the *tpsA* gene (Fillinger *et al*., 2001), and trehalose metabolism involves an acidic, cell wall-localized trehalase required for utilization of exogenous trehalose (d'Enfert and Fontaine, 1997), and a cAMP-regulated neutral trehalase (Nth1) required for trehalose mobilization during spore germination (d'Enfert *et al*., 1999).

We reasoned that several virulence-associated functions in *M.grisea* might involve trehalose mobilization, e.g. germination of conidia and the development of infected cells on the leaf surface, and subsequent plant tissue colonization. Here we report the isolation and characterization of genes encoding biosynthetic and degradative enzymes involved in trehalose metabolism in *M.grisea*. We show that trehalose synthesis in *M.grisea* is mediated by a T6PS-encoding gene, *TPS1*, which is required for production of functional infection structures and primary plant infection. We also demonstrate that trehalose breakdown involves two trehalases; a neutral trehalase, encoded by a gene *NTH1*, which is important for invasive growth in plant cells, and a novel trehalase encoded by *TRE1* which is required for trehalose mobilization during spore germination, but dispensable for pathogenicity. Taken together, our results indicate that trehalose synthe-

© European Molecular Biology Organization

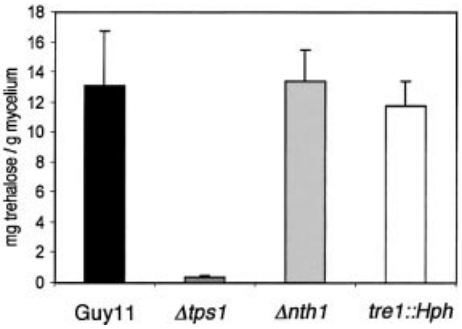


Fig. 1. Bar chart of trehalose levels in mycelium of *M.grisea* strains Guy11, *Δtps1* mutant T-5-13, *Δnth1* mutant T-2-8 and *tre1::Hph* mutant T-8-45. Data points represent the mean value from three experiments. The error bar represents the standard deviation.

sis is required for appressorium-mediated plant infection by a plant pathogenic fungus, while mobilization of stored trehalose is significant only after cuticle penetration.

Results

Trehalose synthesis is required for pathogenicity of M.grisea

To determine the role of trehalose synthesis in pathogenesis of *M.grisea*, we identified a gene encoding T6PS by designing degenerate primers to regions conserved among T6PS genes. A 650 bp product was amplified and used to identify a cDNA and genomic clone. This revealed the presence of a 1587 bp open reading frame (ORF) capable of encoding a 529 amino acid protein of 58.4 kDa. We named the gene *TPS1*, and the putative gene product showed 76% identity to tpsA from *A.niger* and 65% identity to TPS1 from *S.cerevisiae* (DDBJ/EMBL/GenBank accession No. AY148093; see Supplementary data available at *The EMBO Journal* Online). A gene replacement of *TPS1* was carried out. For this, an 8 kb *Pst* I fragment was isolated and a 2.0 kb *Nco*I fragment containing part of the *TPS1* promoter and most of the ORF was removed and replaced with the *Hph* cassette. The resulting vector was introduced into a wild-type rice pathogenic strain of *M.grisea*, Guy11, and 40 transformants were selected. In DNA gel blots, one transformant, T-5-13, had undergone the *Δtps1* gene replacement (see Supplementary data). The putative *Δtps1* mutant T-5-13 was selected and mycelial cultures prepared for measurement of intracellular trehalose. We found that trehalose was almost completely absent from the *Δtps1* mutant, compared with levels observed in Guy11 and either *Δnth1* or *tre1::Hph* mutants (Figure 1).

We investigated the role of *TPS1* in growth, development and virulence of *M.grisea*. The *Δtps1* mutant showed normal mycelial growth and did not respond differently when exposed to heat shock (not shown). Sporulation was dramatically reduced in the *Δtps1* mutant. Guy11 produced a mean of 1.39×10^6 conidia per plate culture ($n = 8$) compared with 3.6×10^3 for *Δtps1* strain T-5-13 ($P < 0.001$). Conidial germination rates and appressorium formation were not significantly affected by the *Δtps1* mutation (not shown). To investigate the role of *TPS1* in pathogenesis, conidial suspensions were sprayed onto seedlings of the susceptible rice cultivar CO-39. A

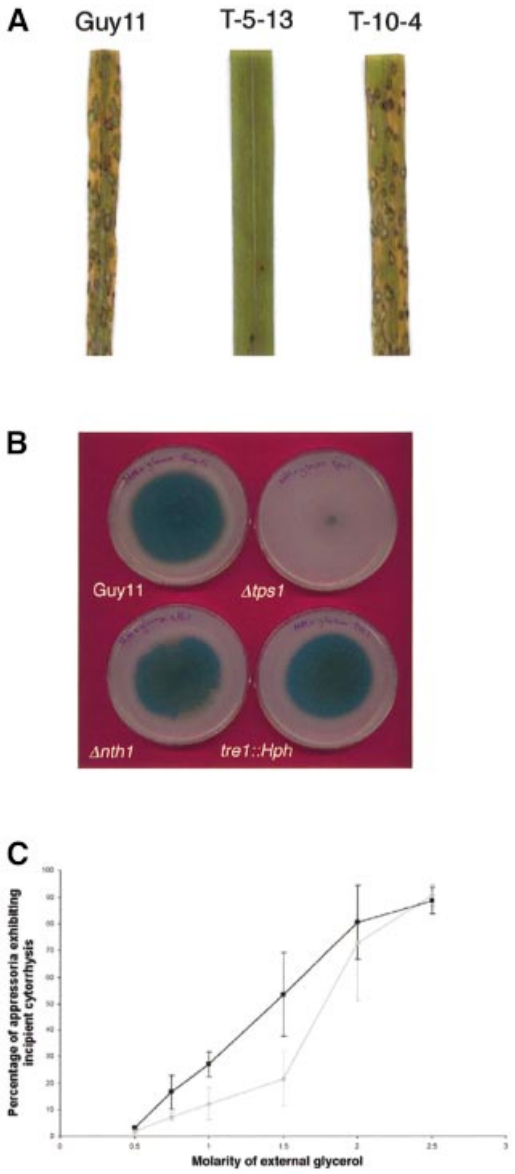


Fig. 2. Phenotypic analysis of *M.grisea Δtps1* mutant T-5-13. (**A**) Virulence phenotype of the *Δtps1* mutant. Seedlings of cv. CO-39 were inoculated with *M.grisea* conidial suspensions of identical concentration (2×10^4 conidia/ml) of strain Guy11 (leaf 1), *Δtps1* mutant T-5-13, and a transformant (T-10-4) of *Δtps1*mutant T-5-13 carrying a single copy insertion of pAJF200, an 8 kb *Pst*I fragment of *TPS1*. Seedlings were incubated for 4 days for development of rice blast disease. (**B**) Growth of *M.grisea* strain Guy11, *Δtps1* mutant T-5-13, *Δnth1* mutant T-2-8 and *tre1::Hph* mutant T-8-45 with glucose as sole carbon source. (**C**) Appressorium turgor of Guy11 (grey diamonds) and *Δtps1* mutant T-5-13 (black squares) was measured by incipient cytorrhysis. The proportion of appressoria that collapsed after exposure to solutions of between 0 and 5.0 M glycerol was recorded. Each data point is the mean of three independent observations. The error bar represents the standard deviation.

consistent 90% reduction in disease lesion development was found in plants inoculated with *Δtps1* compared with Guy11, as shown in Figure 2A (*t*-test, $P < 0.001$). The small number of disease lesions in *Δtps1* infections did not expand during infection or produce sporulating hyphae. The *Δtps1* mutant was also non-pathogenic on barley. The pathogenicity of *Δtps1* mutants could not be restored by adding trehalose or T6P to conidia, prior to inoculation

Table I. Growth of trehalose metabolic mutants of *M.grisea* on different carbon sources and in supplemented growth medium

Growth medium	Guy-11	*Δtps1*	*Δnth1*	*tre:Hph*
Complete medium (CM)[a]	+++[b]	+++	+++	+++
Oatmeal agar	+++	+	+++	+++
CM – glucose	–	–	–	–
CM – nitrate salts	+++	+++	+++	+++
CM – peptone	+++	+++	+++	+++
CM – yeast extract	+++	+++	+++	+++
CM – casamino acids	+++	+++	+++	+++
CM – vitamin solution	+++	+++	+++	+++
CM – yeast extract and casamino acids	+++	+++	+++	+++
CM – yeast extract and peptone	+++	+++	+++	+++
CM – yeast extract and glucose	–	–	–	–
Minimal medium (MM) + glucose[c]	+++	–	+++	+++
MM + trehalose	+++	–	+++	–
MM + fructose	+++	–	+++	+++
MM + maltose	+++	–	+++	+++
MM + sucrose	+++	–	+++	+++
MM + olive oil	++	–	++	++
MM + sodium acetate	++	–	+	+
MM + sodium acetate + glucose	+++	–	+++	+++
MM + mannitol	++	–	+	+
MM + mannitol + glucose	+++	–	+++	+++
MM + galactose	–	–	–	–
MM + glycerol	–	–	–	–
MM + glycerol + glucose	+++	–	+++	+++
MM + ethanol	–	–	–	–
MM + ethanol + glucose	+++	–	+++	+++
MM – carbon source	–	–	–	–
MM + peptone	–	–	–	–
MM + peptone + glucose	+++	+++	+++	+++
MM + yeast extract	–	–	–	–
MM + yeast extract + glucose	+++	+++	+++	+++
MM + peptone + yeast extract + glucose	+++	+++	+++	+++
MM + casamino acids	–	–	–	–
MM + casamino acids + glucose	+++	+++	+++	+++
MM + casamino acids + peptone + glucose	+++	+++	+++	+++
MM + casamino acids + yeast extract + glucose	+++	+++	+++	+++
MM + casamino acids + fructose	+++	+++	+++	+++
MM + casamino acids + sodium acetate	+++	–	+++	+++
MM + casamino acids + olive oil	++	+	++	++
MM + vitamin solution + glucose	+++	–	+++	+++
MM + vitamin solution + yeast extract + glucose	+++	+++	+++	+++

[a]Complete medium is a rich growth medium containing yeast extract, peptone and casamino acids (see Materials and methods for recipe). CM normally contains glucose as the principal carbon source. The medium was supplemented (+) or depleted (–) as shown.
[b]+++ = extensive hyphal growth and sporulation; ++ = reduced growth; + = very poor growth and sporulation; – = no growth/sparse mycelium, no sporulation. All growth tests were carried out on agar-solidified medium and monitored after 12 days incubation at 24°C.
[c]Minimal medium is described in Materials and methods. Supplementations were made of single or multiple carbon sources as indicated (+).

(not shown). Introduction of the *TPS1* gene into *Δtps1* mutant T-5-13 restored its ability to cause disease (Figure 2A). All other *Δtps1* mutant phenotypes were complemented by introduction of *TPS1* (not shown). We conclude that *TPS1* is required for trehalose synthesis in *M.grisea*, and is necessary for rice blast disease.

TPS1 is required for appressorium-mediated cuticle penetration

To determine why *Δtps1* mutants are non-pathogenic, the ability of the *Δtps1* mutant to penetrate intact rice cuticles was measured. Appressoria developed normally in the *Δtps1* mutant, but production of penetration hyphae and rupture of the plant cuticle were significantly decreased from 81 ± 7.2% successful penetrations by Guy11 to 26.6 ± 17.4% in *Δtps1* ($P < 0.001$). Consistent with this, the *Δtps1* mutant did not cause rice blast symptoms when spores were inoculated on intact rice leaves, but was able to produce disease symptoms when the rice cuticle was first removed by abrasion (not shown). Taken together, the experiments indicate that *TPS1* is required for plant infection, but not subsequent growth of the fungus in rice tissue. Plant infection by *M.grisea* is brought about by the appressorium, which develops very high internal turgor to facilitate generation of mechanical force to breach the rice leaf cuticle. Appressorium turgor can be measured using an incipient cytorrhysis assay which uses hyperosmotic concentrations of a solute to collapse appressoria, thereby allowing estimation of their internal solute concentration and turgor (Howard *et al*., 1991; de Jong *et al*., 1997). To carry out this assay, appressoria were allowed to form on a hydrophobic plastic surface and then incubated in glycerol solutions of varying concentration (Figure 2C). We found that 1.5 M glycerol was sufficient to collapse 21.8 ± 10.4% of appressoria of Guy11 and 53.4 ± 15.7% of *Δtps1* appressoria, indicating a significant decrease in turgor in

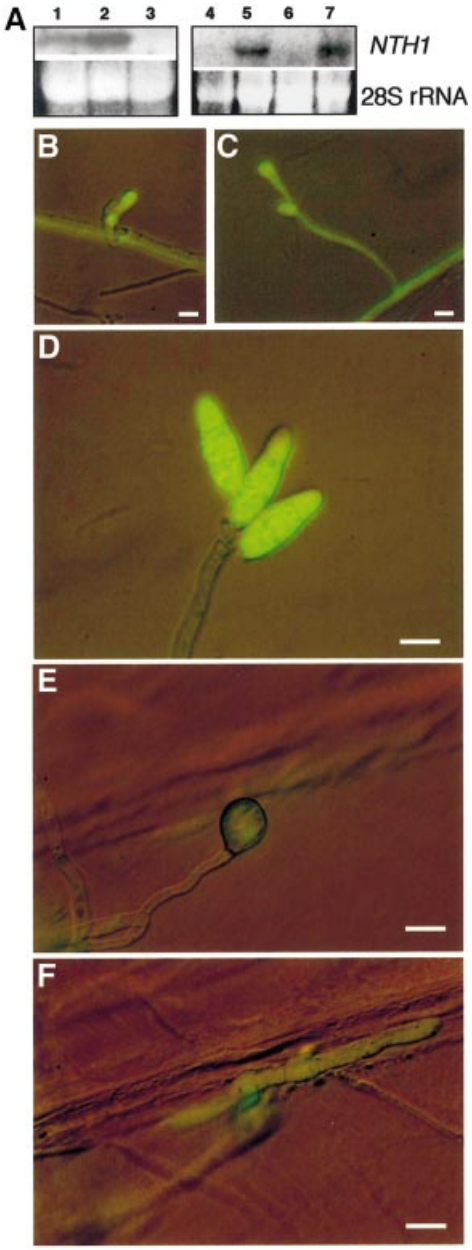


Fig. 3. Expression of *M.grisea NTH1* during conidiogenesis, plant infection and in response to hyperosmotic stress. (**A**) RNA was extracted from a non-conidiating culture of *M.grisea* strain Guy11 (lane 1), a conidiating culture of Guy11 (lane 2) and a conidiating culture of *nth1* mutant E508.R.6 (lane 3). RNA was also extracted from hyphal cultures of *M.grisea* grown in iso-osmotic media, CM (lanes 4 and 6), or subjected to acute hyperosmotic stress in CM + 0.5 M NaCl for 24 h (lanes 5 and 7). RNA was from strain Guy11 (lanes 4 and 5) or *Δosm1* mutant JH73 (lanes 6 and 7). RNA gel blots were probed with *NTH1*. The 28S rRNA is shown as a loading standard. (**B**) Spatial expression of *NTH1*. Early conidiogenesis initiated from a foot cell in *NTH1(p)::sGFP::Hph* transformant T-4-11. (**C**) Conidial initials forming at the end of a conidiophore. (**D**) Mature conidiophore with three sympodially arrayed conidia. (**E**) Germ tube and appressorium on barley epidermis. (**F**) An invasive hypha entering an epidermal cell from the base of the same appressorium. Bar in all panels = 10 μm.

the *Δtps1* mutant ($P = 0.0074$). Turgor was not restored by adding trehalose or T6P to germinating conidia of *Δtps1* (not shown). We conclude that *TPS1* is required for appressorium-mediated cuticle penetration by *M.grisea*.

Fig. 4. Virulence phenotype of *M.grisea Δnth1* mutants. Seedlings of rice cultivar CO-39 were inoculated with *M.grisea* conidial suspensions of identical concentration (2×10^4 conidia/ml) of strain Guy11 (**A**), and *Δnth1* mutants T-2-8, T-2-30 and T-2-37 (**B**–**D**). Seedlings were incubated for 4 days for development of disease.

M.grisea TPS1 is required for growth on glucose

In *S.cerevisiae*, T6PS is required for regulation of glucose intake into glycolysis (Thevelein and Hohmann, 1995). As a consequence, yeast *Δtps1* mutants are unable to grow on glucose. Conversely, in the filamentous fungus *A.nidulans*, *tpsA* may play a role in regulating levels of sugar-phosphates, but mutants lacking T6PS still grow on glucose (Fillinger *et al*., 2001). To determine whether *M.grisea TPS1* is required for glycolytic regulation, we incubated the *Δtps1* mutant on minimal growth medium with glucose as sole carbon source (Figure 2B). The *Δtps1* mutant was unable to grow on glucose or other simple sugars including fructose, sucrose, maltose and trehalose (Table I). In addition, unlike yeast T6PS mutants, *M.grisea Δtps1* failed to grow on acetate or fatty acids, indicating that gluconeogenesis is also affected by the absence of the T6PS. In rich growth media, we observed that *Δtps1* mutants were able to utilize glucose, but only in the presence of a rich source of amino acids, provided either from yeast extract, peptone or casamino acids. Under these conditions, *Δtps1* mutants were still unable to utilize acetate as a carbon source (Table I). We conclude that *M.grisea TPS1* is required for growth on a range of rapidly fermentable carbon sources, and may be required for regulation of glycolysis and gluconeogenesis.

Isolation of NTH1 encoding a neutral trehalase from M.grisea

To investigate how stored trehalose in *M.grisea* is mobilized, we identified a gene encoding the intracellular regulated form of trehalase. A previous study to identify *M.grisea* genes involved in virulence using insertional mutagenesis had revealed a gene called *PTH9*, which encodes a protein showing similarity to neutral trehalase (Sweigard *et al*., 1998). We obtained the *pth9* insertion mutant E508-R-6 and a *PTH9* genomic clone (kindly provided by Dr J.A.Sweigard, DuPont Company, Wilmington, DE). DNA sequencing defined a 2211 bp ORF encoding a 736 amino acid protein of 84.6 kDa, showing 81% identity to the treB neutral trehalase enzyme from *N.crassa* and 68% identity to treB from *A.nidulans* (DDBJ/EMBL/GenBank accession No. AY148092; see Supplementary data). We named the gene *NTH1* for neutral trehalase. The NTH1 protein sequence contains a

consensus phosphorylation site for cAMP-dependent protein kinase A (PKA) and a putative Ca^{2+}-binding site, indicating that the gene encodes a regulated form of trehalase. To investigate *NTH1* expression, RNA gel blots showed that the 2.7 kb *NTH1* transcript was 4-fold more abundant during conidiogenesis, than in vegetative hyphae. *NTH1* was also expressed abundantly in mycelium after exposure to 0.5 M NaCl for 24 h. The same response was observed after hyperosmotic stress using sorbitol (not shown) and was independent of the presence of the osmoregulatory mitogen-activated protein (MAP) kinase gene *OSM1* (Dixon *et al*., 1999), as shown in Figure 3A. Spatial expression of *NTH1* was determined by fusing a 2.5 kb *NTH1* promoter fragment upstream of the green fluorescent protein (GFP)-encoding gene *sGFP* (Chiu *et al*., 1996) and introducing the plasmid into *M.grisea*. Transformants containing a single copy of *NTH1(p):sGFP* were examined by epifluorescence microscopy (Figure 3B). *NTH1(p):sGFP* expression occurred during initial stages of conidiation when conidiophores develop from a foot cell and differentiate to produce a sympodial array of conidia (Figure 3B–D). Expression was also observed in invasive hyphae within plant tissue, indicating that expression of *NTH1* occurs after cuticle penetration (Figure 3E–F). Sequence analysis of the *NTH1* promoter revealed putative binding sites for homologues of the stuA and abaA transcriptional activators known to regulate conidiogenesis in *A.nidulans* (Timberlake, 1993) and three consensus stress response elements (STREs) found in genes expressed during the multistress response in yeast (Schuller *et al*., 1994). We conclude that *NTH1* is expressed during *M.grisea* sporulation, plant infection and in response to environmental stress.

NTH1 encodes a virulence factor for rice blast disease

The *pth9* insertional mutant E508-R-6 contained a 4.0 kb plasmid insertion in the *NTH1* ORF, but theoretically was capable of producing a truncated *NTH1* trehalase (Figure 4A and B), although RNA gel blots suggested that the mutant did not produce an *NTH1* transcript (Figure 3A). To ensure generation of a null mutant, a gene replacement was carried out by deleting 2.4 kb of *NTH1* and replacing this with a hygromycin resistance cassette. DNA gel blots identified three transformants where the gene replacement had occurred from 38 selected (see Supplementary data available at *The EMBO Journal* Online). The three *Δnth1* mutants T-2-8, T-2-30 and T-2-37 were not affected in hyphal growth, response to heat stress or the temporal dynamics of spore germination (not shown). The *Δnth1* mutants did show a significant decrease in sporulation, producing ~50% of the number of conidia in the wild-type Guy11 (t = 8.77, $P < 0.001$, df = 30). To test the effect of *Δnth1* deletion on rice blast disease, conidial suspensions of three mutants and Guy11 (at identical concentrations) were sprayed onto seedlings of rice cultivar CO-39. The *Δnth1* mutants all produced significantly fewer disease lesions than Guy11 (Figure 4). The *Δnth1* mutants showed lesion densities that were 24 ± 16% of those produced by Guy11 (t-tests of lesion densities were significant at $P < 0.001$, n = 100 leaves). The pathogenicity of *Δnth1* mutants on barley seedlings showed a similar reduction. To determine the likely cause of the pathogenicity defect,

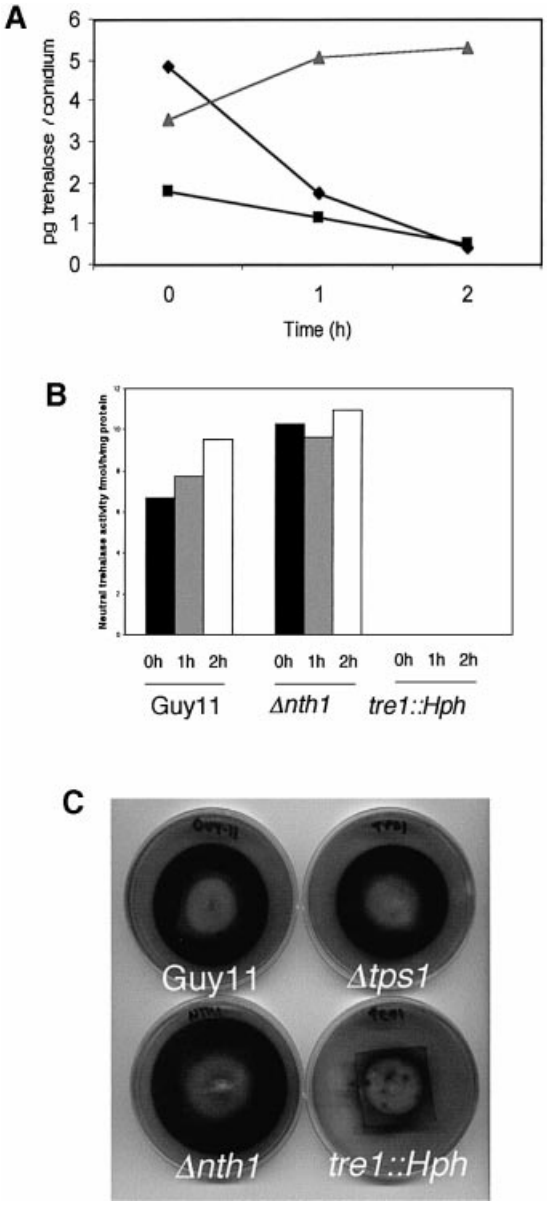


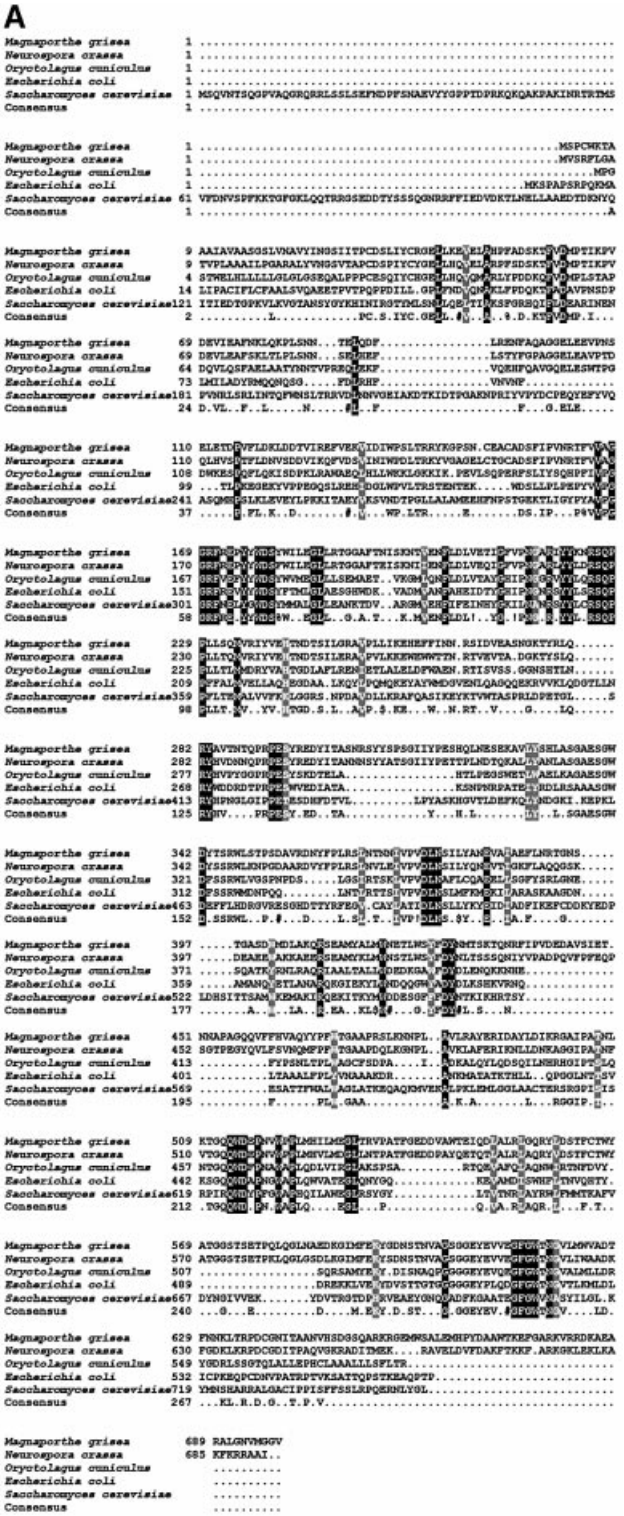
Fig. 5. Trehalose mobilization and neutral trehalase activity during conidial germination of *M.grisea.* (**A**) Line graph of trehalose levels in conidia of Guy11 (diamonds), *Δnth1* mutant T-2-8 (squares) and *tre1::Hph* mutant T-8-45 (triangles) at three intervals during germination. Data points represent the mean of three replications of the experiment. (**B**) Bar chart of neutral trehalase activity in conidia in the same strains at identical intervals. Each bar represents the mean of three replicate assays. (**C**) Neutral trehalase activity in vegetative hyphae of *M.grisea* assayed using an overlay test (see Materials and methods). Cultures of Guy11, *Δnth1* mutant T-2-8, *tre1::Hph* mutant T-8-45 and *Δtps1* mutant T-5-13 were grown for 6 days prior to assay. All experiments were repeated at least three times with similar results.

we assayed cuticle penetration and appressorium turgor generation (see Supplementary data). No significant differences were observed compared with the wild-type strain, indicating that the ability to infect plants was not compromised by the *Δnth1* mutation. Instead, we observed that proliferation of invasive hyphae was not as rapid in *Δnth1* mutants as in Guy11 (not shown). Re-introduction of *NTH1* into a *Δnth1* mutant restored pathogenicity and conidiogenesis to wild-type levels (not shown). We conclude that *NTH1* is required by *M.grisea* to generate severe rice blast symptoms.

TRE1 encodes the major trehalase activity in germlings and hyphae of M.grisea

The temporal expression pattern of *NTH1* and reduction in conidiation in *Δnth1* mutants pointed to a potential role for *NTH1* in mobilization of trehalose during spore germination. To investigate this, we measured the amount of trehalose in conidia and germlings of *M.grisea* at three different times during germination (Figure 5A). In Guy11, trehalose was mobilized rapidly during germination, and almost completely degraded after 2 h. A similar pattern of trehalose degradation was observed in *Δnth1* mutant T-2-8, although the initial amount of trehalose in conidia was less than in Guy11. The pattern of trehalose degradation in *Δnth1* mutants suggested to us that *M.grisea* must possess another intracellular trehalase activity, and we therefore assayed neutral trehalase activity at the same times during conidial germination. The *Δnth1* mutant showed no significant difference in neutral trehalase activity during conidial germination when compared with Guy11 (Figure 5B), confirming the presence of a second trehalase in *M.grisea*.

To identify the second trehalase, we used a bioinformatic approach and scanned all available DNA sequence information from *M.grisea* for homology to eukaryotic trehalase-encoding enzymes. We found a small fragment of a putative trehalase-encoding gene in a sequence from a bacterial artificial chromosome, used in the *M.grisea* genome mapping effort (Zhu *et al*., 1999). We amplified a corresponding 504 bp gene fragment and used this to isolate a cDNA and genomic clone spanning the locus. DNA sequencing revealed a 1914 bp ORF interrupted by four putative introns, capable of encoding a 698 amino acid protein (Figure 6A) with a putative signal peptide of 22 amino acids at the N-terminus. We named the gene *TRE1* (trehalase-encoding). The putative *TRE1* product showed 33% identity to the human and mouse TreA trehalases, but was distinct from both neutral and acidic trehalases previously identified from fungi (Figure 6B). Scanning the publicly available genome sequence of *N.crassa* revealed a gene showing 66% identity to *TRE1*, but no homologous sequence was observed in the *Aspergillus fumigatus* draft genome sequence. Thus, only closely related filamentous fungi (*N.crassa* and *M.grisea* are both pyrenomycetes) appear to possess this novel form of trehalase. To determine the cellular location of the enzyme, we produced a *TRE1:GFP* gene fusion by



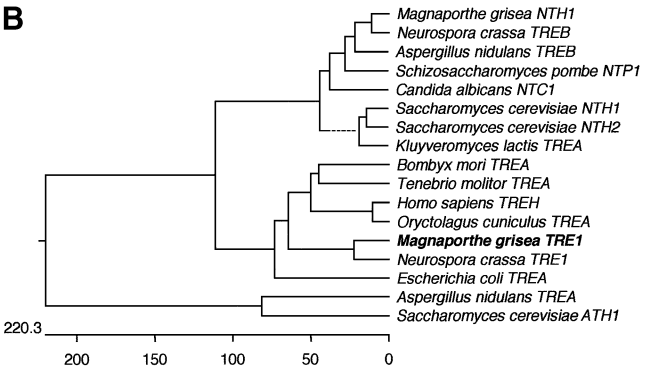


Fig. 6. Predicted amino acid sequence of the *M.grisea TRE1* gene product. (**A**) Sequences were aligned using Clustal W (Thompson *et al*., 1994). The *TRE1* gene product is aligned with sequences of *N.crassa* hypothetical protein NCU00943.1 (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/), rabbit TreA (P19823), *Escherichia coli treA* (P13482) and *S.cerevisiae NTH1* (P321356). Identical amino acids are highlighted on a black background, and similar amino acids on a light grey background. Gaps are indicated by dashes. *M.grisea TRE1* has DDBJ/EMBL/GenBank accession No. AF543465. (**B**) Dendrogram indicating the relationship of *TRE1* to previously identified trehalase gene products. Genetic distance is proportional to the length of the horizontal line.

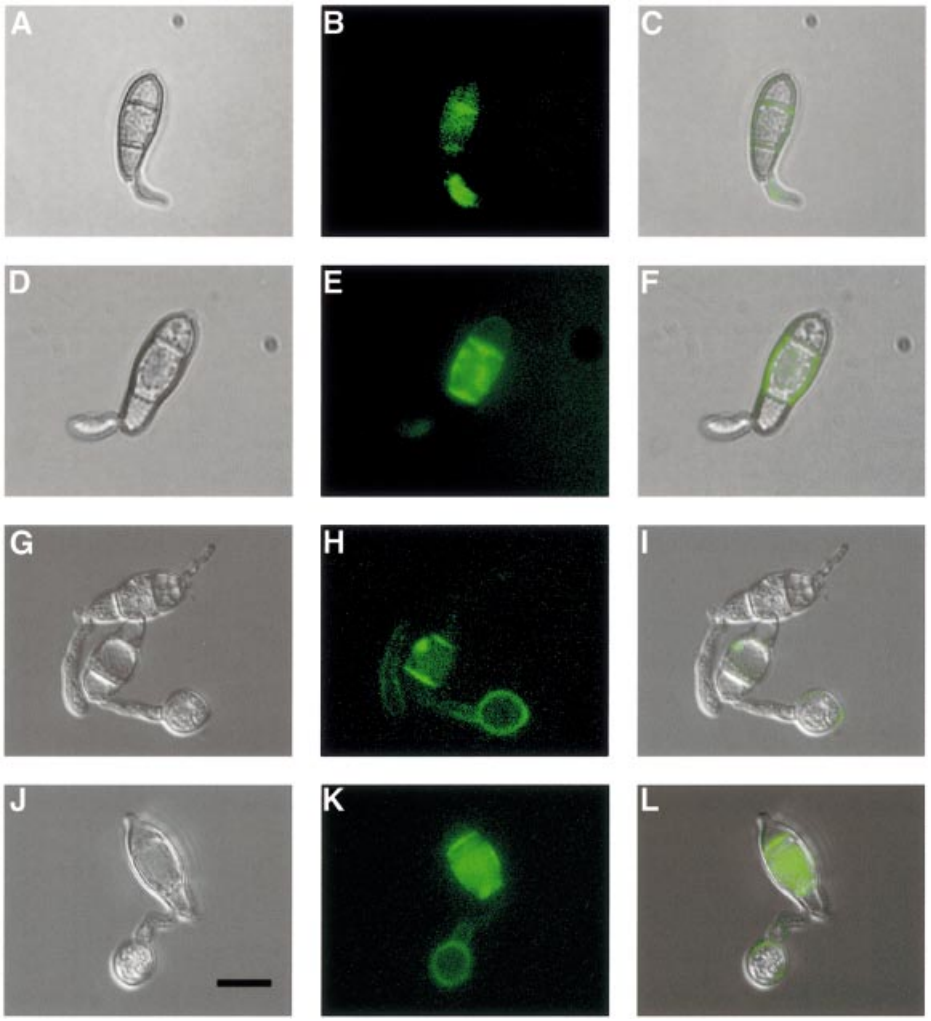


Fig. 7. Localization of *TRE1* trehalase during infection-related development of *M.grisea*. Conidia of Guy11 *TRE1:sGFP* transformant T-9-14 were allowed to form appressoria on plastic surfaces. Bright-field images are shown in the left-hand panels, epifluorescence images in the centre and merged images in the right-hand panels. (**A**–**C**) A three-celled conidium undergoing germination and germ tube extension 2 h after inoculation; TRE1:GFP accumulates in septa and germ tube apex. (**D**–**F**) A germ tube extension with septal localization of TRE1:GFP, 2 h after inoculation (**G**–**I**) A germ tube undergoing hooking and appressorium formation after 6 h. The appressorium is the round structure at the end of the fungal germ tube. (**J**–**L**) Appressorium maturation with wall localization of TRE1:GFP after 7 h. Bar = 10 μm.

introducing the *sGFP* allele at the C-terminus of the *TRE1* ORF. The gene fusion was transformed into Guy11, and transformants containing a single copy of the *TRE1:GFP* fusion were selected (Figure 7). TRE1:GFP accumulates in the septa of germinating conidia (Figure 7A–F), the germ tube apex and the walls of developing appressoria (Figure 7H and K). The temporal pattern of TRE1:GFP accumulation was consistent with RNA gel blots which showed *TRE1* expression during the early stages of conidial germination (not shown). A targeted disruption of *TRE1* utilized an adaptation of an *in vitro* transposon tagging procedure previously described (Hamer *et al*., 2001). We modified the Tn*7* transposable element of pGPS3 (New England Biolabs), by introducing the hygromycin B resistance gene cassette. The modified transposable element was mobilized *in vitro*, allowing us to introduce transposon insertions throughout a cloned 8 kb genomic DNA fragment spanning the *TRE1* locus. A gene disruption construct containing the 3 kb GPS-HYG transposon insertion at the 5′ end of the *TRE1* coding region was introduced into *M.grisea* strain Guy11, and 56 hygromycin-resistant transformants were selected. Twenty-seven of these (48%) showed the presence of the gene disruption, with the 8 kb *TRE1* locus replaced by the 11 kb *tre1::Hph* insertion allele (see Supplementary data).

Trehalose mobilization was measured in germinating conidia of a *tre1::Hph* mutant T-8-45 (Figure 5A). Conidial trehalose levels stayed constant throughout germination, indicating that trehalose degradation does not occur in a *tre1::Hph* mutant (three mutants showed the same pattern). Consistent with this, we were unable to detect any trehalase activity in *tre1::Hph* mutants during conidial germination (Figure 5B). We also developed a plate assay for visualizing trehalase activity which results in production of a brown precipitate in the presence of trehalase. The assay showed that trehalase activity was absent from mycelium of the *tre1::Hph* mutant and suggested that *TRE1*-encoded trehalase is secreted because activity was observed as a dark halo surrounding hyphal cultures (Figure 5C). Furthermore, *tre1::Hph* mutants

failed to grow on trehalose as a sole carbon source (Figure 8), and *TRE1* showed enhanced expression in response to trehalose (not shown). *TRE1* therefore encodes a trehalase with attributes of both acidic trehalase, which has been shown to be required for trehalose utilization in *S.cerevisiae* and *A.nidulans*, and neutral trehalase, which is required for intracellular trehalose mobilization in both fungi (Destruelle *et al*., 1995; Nwaka *et al*., 1995; d'Enfert and Fontaine, 1997; d'Enfert *et al*., 1999). Infection assays on rice showed that *tre1::Hph* mutants were as virulent as the wild-type Guy11 (Figure 8). *TRE1*-encoded trehalase activity is thus dispensable for fungal pathogenicity.

Discussion

Trehalose is a versatile compound that can fulfil a wide variety of functions in cells, acting as a reserve carbohydrate for survival during stress, a membrane protectant, a compatible solute, or acting to stabilize protein structures during adverse conditions (Thevelein, 1984). In this report, we have shown that the main trehalose biosynthetic enzyme T6PS, encoded by *TPS1*, is required for fungal virulence, affecting the ability of the fungus to breach the plant cuticle, while a trehalase-encoding gene *NTH1* has an effect on subsequent stages of disease development. Taken together, these results point to a significant role for trehalose metabolism in allowing the fungus to be able to cause plant disease.

This study is the first report concerning trehalose metabolism in a plant pathogenic fungus, and several features appear distinct from previous analyses of *S.cerevisiae* and filamentous fungi such as *A.nidulans*. Trehalose synthesis in *M.grisea* requires the T6PS-encoding gene *TPS1* which is necessary for efficient sporulation and growth on glucose. In this way, *M.grisea TPS1* resembles the *S.cerevisiae TPS1* gene which is needed for ascospore production and survival on glucose (Bell *et al*., 1998). In contrast, the *A.nidulans ΔtpsA* mutants grow normally on glucose (Fillinger *et al.*, 2001). In yeast, three different models have been proposed to account for the role of Tps1 in glycolytic regulation (Arguelles, 2000). Trehalose synthesis may be a means of removing excess sugar-phosphates, thereby recycling phosphate for use by glyceraldehyde-3-phosphate dehydrogenase at a later stage of glycolysis. Alternatively, T6P, the product of T6PS, may inhibit hexokinase II activity. Inhibition of hexokinase occurs *in vitro* using physiologically relevant concentrations of T6P, and would provide a means of limiting glucose-6-phosphate entry into glycolysis (Bell *et al*., 1998). Finally, Tps1 may directly affect sugar uptake and phosphorylation (Thevelein and Hohmann, 1995). Interestingly, *M.grisea* appears to integrate trehalose synthesis with glycolytic regulation in a similar manner to *S.cerevisiae*, but also requires T6PS in order to utilize acetate or lipid as carbon sources. Activation of the glyoxylate cycle, or subsequent production of glucose via gluconeogenesis, may therefore be disrupted by the absence of T6PS in *M.grisea.* The inability of *Δtps1* mutants of *M.grisea* to grow on glucose could be overcome by addition of amino acids to the medium, suggesting that synthesis of complex organic nitrogen compounds might also be affected by lack of T6PS. However, the presence of amino acids did not restore the ability of *Δtps1* mutants to grow on acetate, indicating that gluconeogenesis is affected by loss of T6PS regardless of whether the fungus is utilizing nitrate or amino acids as a nitrogen source.

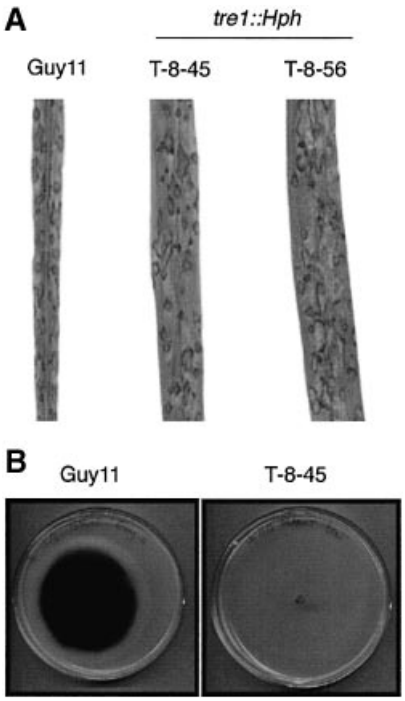


Fig. 8. Phenotypic analysis of *M.grisea tre1* mutants. (**A**) Pathogenicity of *M.grisea tre1* mutants. Seedlings of rice cultivar CO-39 were inoculated with *M.grisea* conidial suspensions of Guy11 and *tre1::Hph* mutants T-8-45 and T-8-56. Seedlings were incubated for 4 days for development of disease. (**B**) Growth of *M.grisea* strain Guy11 and *tre1::Hph* mutant T-8-45 with trehalose as the sole carbon source.

The mechanism of trehalose degradation in *M.grisea* differs substantially from that in *S.cerevisiae* and *A.nidulans* due to the presence of the novel *TRE1*-encoded trehalase. This trehalase is distinct from both neutral and acidic trehalases and is required both for growth on trehalose and mobilization of intracellular trehalose. Its localization in the cell wall is consistent with the presumed location of acidic trehalase in *A.nidulans* and *N.crassa*, but does not account for its role in intracellular trehalase activity. It may be that sufficient cytoplasmic accumulation of TRE1 occurs during spore germination, or that the localization of TRE1 in septa may be associated with this function. Although *NTH1* is highly expressed during spore germination, its product does not appear to contribute to neutral trehalase activity at this time, and may be activated at a later stage of invasive growth. The presence of a consensus PKA phosphorylation motif in NTH1 and the known importance of cAMP-dependent signalling to disease progression in *M.grisea* (Talbot and Foster, 2001) provide a possible mechanism for post-translational activation of neutral trehalase after plant infection.

The role of trehalose metabolism in rice blast disease

Plant infection by *M.grisea* involves the action of a specialized infection cell, the appressorium, a structure widely utilized by pathogenic fungi to invade the tissues of their hosts (Tucker and Talbot, 2001). Turgor generation in appressoria results from accumulation of up to 3 M

glycerol in the appressorium (de Jong *et al*., 1997) and, therefore, mobilization of storage reserves is likely to be critical for appressorium function. Our results indicate that *TPS1* is required for appressorium function and cuticle penetration. The simplest explanation for this observation is that trehalose is used as a reserve carbohydrate that contributes to appressorium turgor by acting as a compatible solute itself, or being converted to the pool of intracellular glycerol in the appressorium. Trehalose could also act as an accessory compatible solute or membrane protectant to allow appressoria to withstand high turgor. Trehalose possesses physico-chemical properties including high hydrophilicity and chemical stability that might allow cellular proteins to operate effectively within mature appressoria (Arguelles, 2000). The addition of trehalose or T6P to germinating conidia of a *Δtps1* mutant, however, did not restore pathogenicity or appressorium turgor. Furthermore, trehalose degradation during conidial germination, which is mediated by *TRE1*, is clearly not required for appressorium function because *tre1* mutants are fully pathogenic. When considered together, these results suggest that although the presence of *TPS1* is required for appressorium function, the generation and utilization of trehalose within the infection cell may not be as significant.

An alternative role for *TPS1* in pathogenicity of *M.grisea*, which we favour, arises because of its role in regulation of glycolysis and gluconeogenesis. During appressorium turgor generation by *M.grisea*, it is likely that the major route for glycerol biosynthesis is via lipid metabolism (Thines *et al*., 2000). Lipid bodies move to the appressorium during maturation and are degraded by triacylglycerol lipase during turgor generation. Appressoria develop on the leaf surface in the absence of external nutrients. Under these glucose- and nitrogen-deficient conditions, the metabolism of acetyl-CoA, produced from fatty acid β-oxidation via the glyoxylate cycle and gluconeogenesis, may be essential for the fungus to carry out growth functions such as cell wall biosynthesis. The absence of TPS1 may mis-regulate the balance between glycolysis and gluconeogenesis during turgor generation. The fact that *M.grisea Δtps1* mutants cannot grow on acetate or fatty acids is consistent with such a function and is distinct from the phenotype observed in *S.cerevisiae tps1* mutants (Arguelles, 2000).

The role of *NTH1* in pathogenicity is very different from that of *TPS1*. Although disease lesion number was reduced in infections by *Δnth1*, there was no reduction in the rate of cuticle penetration. Rather, the ability of the mutant to grow once it had entered the plant appeared to be impaired. The reduction in symptom development may arise due to inability of the fungus to proliferate effectively in plant tissue and withstand desiccation, which is a visible consequence of tissue necrosis during rice blast infections. The reduction in virulence of *Δnth1* was clearly not due to a reduction in trehalose mobilization during pre-penetration stage development, because of the importance of *TRE1* in these processes and its dispensability for pathogenicity.

In summary, trehalose metabolism plays a pivotal role in the ability of *M.grisea* to cause disease. Trehalose biosynthesis, either directly or via a regulatory effect, is necessary for initial plant infection, while trehalose mobilization is involved in virulence-associated functions that follow host colonization. Deletion of *TPS1* in the human pathogenic fungus *Candida albicans* also impairs its ability to cause disease (Zaragoza *et al*., 1998), highlighting the potential widespread significance of trehalose metabolism to microbial pathogenesis.

Materials and methods

Fungal strains, growth conditions and DNA analysis

Magnaporthe grisea maintenance, media composition, DNA extraction, RNA extraction and transformation were all as described previously (Talbot *et al*., 1993). Minimal medium (MM) is 6 g/l $NaNO_3$, 0.52 g/l KCl, 0152 g/l $MgSO_4{\cdot}7H_2O$, 1.52 g of KH_2PO_4, 0.001% thiamine, 0.1% trace elements supplemented with 10 g/l glucose or an equivalent amount of alternative carbon sources used. Complete medium (CM) is MM supplemented with 2 g/l peptone, 1 g/l yeast extract and 1 g/l casamino acids. All molecular biology methods were performed using standard procedures (Sambrook *et al*., 1989). Sequence alignments were performed using the Clustal_W program (Thompson *et al*., 1994). Dendrograms were made by applying neighbour-joining methods (Saitou and Nei, 1987) to the distance matrix generated by Clustal_W in Phylip format (Felsenstein, 1989). The calculated phylogenetic tree was viewed using the program TreeView (Page, 1996).

Identification of NTH1, TPS1 and TRE1

To isolate *TPS1*, degenerate primers were designed based on the amino acid sequences of known fungal T6PS-encoding genes. The nucleotide sequences of the primers were as follows: PTPS1, 5′-YTNTGGCC-NYTNTTYCAYTAY-3′; PTPS2, 5′-GGTNCANCAYTAYCAYYTNA-TG-3′; PTPS3, 5′-NTWYTGRTAYTCYTCNACRTG-3′; and PTPS4, 5′-GNGGNAYNCCYTTDATRTARTC-3′.

The 650 bp amplicon was cloned into pGEM-T (Promega) and used as a probe to obtain the corresponding genomic and cDNA clones. To isolate *NTH1*, three restriction fragments were subcloned from a 14 kb *Eco*RV fragment in pCB1378 and sequenced (Sweigard *et al*., 1998). *TRE1* was identified from a BAC-end sequence during the *M.grisea* genome mapping project (Zhu *et al*., 1999). Primers were designed to amplify this sequence from genomic DNA: PTREA1, 5′-CAACATCTCCAAGA-ACACCGT-3′; and PTREA2, 5′-GCCTTTTTGGACTCGGTAAC-3′.

The resulting 500 bp amplicon was sequenced and used to screen *M.grisea* cDNA and genomic libraries. An 8 kb *Pst*I–*Sac*I fragment containing the entire *TRE1* locus was selected.

Construction of targeted gene replacement vectors

All gene replacements employed the *Hph* gene which encodes hygromycin phosphotransferase under the control of the *A.nidulans* TrpC promoter (Carroll *et al*., 1994). Gene replacement of *TPS1* was carried out by selecting and sequencing an 8 kb *Pst*I fragment spanning the locus. A 2.0 kb *Nco*I fragment containing the 5′ end and the majority of the *TPS1* coding region was replaced with an *Afl*III-linked *Hph* cassette produced by PCR using primers HYGAFLIII 1, 5′-CCAGACATGTCACGACGTTGTAAAACGACGG-3′; and HYGAF-LIII 2, 5′-CCAGACATGTGTCGACTCTAGAGGATCCCC-3′ using pCB1004 (Carroll *et al*., 1994) as a template. The resulting plasmid was digested with *Pst*I and used to transform *M.grisea*. Complementation of *Δtps1* was carried out by cloning the 8 kb *TPS1 Pst*I fragment into pCB1578 and selecting sulfonylurea-resistant transformants. Transformants containing a single integration of the *TPS1* were selected. For replacement of *NTH1*, a 2.4 kb *Apa*I fragment was excised from pCB1378 and the vector re-circularized to create pAJF12. The *Hph* gene from pCB1003 (Carroll *et al*., 1994) was used a template for amplification of an *Apa*I-linked *Hph* fragment using the universal forward primer and primer HYGAPA1, 5′-AGTCAGGGGCCCAATTAACCCTCACTATAAAGG-G-3′. The *Apa*I-linked *Hph* cassette was ligated to *Apa* I-digested pAJF12. The resulting vector pAJF14 was partially digested with *Nco*I to release a 9 kb fragment which was introduced into Guy11. Complementation of *Δnth1* required cloning of the 14 kb *Eco*RV fragment from pCB1378 into pCB1578, which contains a sulfonylurea resistance selectable marker (Carroll *et al*., 1994). The resulting plasmid pAJF87 was introduced into *Δnth1* mutant T-2-8, and transformants containing a single integrated copy of the plasmid were selected.

TRE1 was disrupted using an adaptation of the TAG-KO method (Hamer *et al*, 2001). The kanamycin resistance gene was excised from

pGPS3 (New England Biolabs) and the vector ligated to a PCR amplicon containing the *Hph* gene and *Cat* chloramphenicol resistance gene from pCB1004. Primers used to amplify the *Hph Cat* PCR product were: HYGCAM1, 5′-CGCGGATCCGACGTTGATCGGCACGTAAGAGG-3′; and HYGCAM2, 5′-CGCGGATCCGAAGAACGTTTTCCAATGA-TGATGGCAC-3′.

The resulting plasmid, pGPS-HYG, contains the selectable markers between Tn*7*-based transposon ends and therefore represents an immobilized transposable element (Craig, 1996). The element was mobilized using TnsABC Transposase complex *in vitro* (New England Biolabs). An 8 kb *Pst*I–*Sac* I fragment of *TRE1*, pTRE-G, was used as the target. An insertion was found within the coding regions of *TRE1*, close to the 5′ end of the ORF, and confirmed by sequencing. This plasmid, pTRE1-GPS-Hyg, was digested with *Pst*I and *Sac*I to liberate an 11 kb linear gene disruption cassette, which was transformed into Guy11.

Measurement of intracellular trehalose and neutral trehalase activity

To assay trehalose content of conidia, a suspension of conidia was harvested from 10-day-old plate cultures of *M.grisea* and allowed to germinate at a concentration of 1×10^6 conidia/ml on plastic coverslips at 24°C for 1 or 2 h. Conidia were then recovered by centrifugation and re-suspended in 500 µl of water. The suspension was boiled for 5 min and sonicated before removal of cell debris by centrifugation. A 60 µl aliquot was added to 60 µl of 0.1 M sodium citrate buffer. Duplicate samples were incubated in either the presence or absence of 4 µl porcine kidney acidic trehalase (Sigma) at 37°C overnight. The concentration of glucose in each sample was assayed using a commercial kit (Roche). A plate assay for neutral trehalase was developed for *M.grisea* from the enzymatic overlay test of Kopp *et al*. (1993). Plate cultures were grown for 3 days. A filter paper disc (Whatman 3 mm) was cut to the size of a Petri dish and sterilized, before being placed on the culture and incubated for 3 days. The assay was then as described by Kopp *et al*. (1993) except that incubations were carried out at 24°C rather than 30°C. The colour change is due to hydrolysis of trehalose to glucose by trehalase from the fungus; the resulting glucose is hydrolysed to gluconic acid and hydrogen peroxide by glucose oxidase. A peroxidase–hydrogen peroxide complex then oxidizes the chromogen *o*-diansidine to cause the colour change.

To assay neutral trehalase in conidia, a suspension of conidia was re-suspended in 15 ml of extraction buffer [50 mM Tris–HCl pH 7.0 and EDTA-free protease inhibitor cocktail tablets (Roche)]. The suspension was disrupted using a Bead-Beater™ (Biospec Products). A 60 µl aliquot of supernatant was mixed with 60 µl of assay buffer (50 mM Tris pH 7.0, 10 mM $CaCl_2$, 18 mM $MgCl_2$, 1 mM ATP, 20 µM cAMP) in the presence or absence of 40 mM trehalose. Reaction samples were incubated at 30°C for 2 h and then boiled for 5 min. Glucose concentration was determined using a commercial kit (Roche). The concentration of protein was determined using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad).

Gene expression studies

The *NTH1*p-*sGFP* plasmid was constructed by amplification of the 5′ end of *NTH1* using primer NTHGFP, 5′-GAGCTTCGCCATGGTGA-ATGACTT-3′, and the vector-specific T7 primer (Stratagene). The 2.6 kb promoter fragment was cloned into pGEM-T (Promega) to give pAJF18. The *sGFP* allele (Chiu *et al*., 1996) carrying the *A.nidulans trpC* terminator (Punt *et al*., 1987) was excised as an *Nco*I–*Sac*I fragment from plasmid pMJK-80 (Kershaw *et al*., 1998) and ligated to pAJF18. The resulting plasmid, pAJF19, was linearized and ligated to *Apa*I-linked *Hph* to create pAJF20. The in-frame fusion of the *NTH1* promoter with the *sGFP* gene was checked by DNA sequencing. Transformants containing a single integration of pAJF20 were selected. Epifluoresence microscopy was used to detect GFP using a Nikon Optiphot-2 microscope (Swindon, UK). The *TRE1:sGFP* gene fusion was made by amplifying a 4.8 kb fragment of *TRE1* containing 2.8 kb of upstream promoter and the entire protein-coding sequence using the vector-specific T7 primer (Stratagene) and primer GFPTRE: 5′-TTTTCCATGGCACCTCCCATGACGTT-CCC-3′. This primer adds an *Nco*I site (underlined) at the last codon of *TRE1*. The amplicon was digested with *Sac*I and *Nco*I and cloned into *Sac*I–*Nco*I-digested pAJF20 from which the *NTH1* promoter sequence had been removed. This provided an in-frame fusion of *TRE1*, under control of its native promoter, to *sGFP*. The entire insert was sequenced. The *TRE1:sGFP* construct was transformed into Guy11, and transformants carrying a single insertion were selected. A total of four *TRE1:sGFP* transformants were characterized, with identical results.

Plant infections assays

Plant infections were as described previously (Talbot *et al*., 1993). Conidial suspensions were diluted in 0.1% gelatin to 1×10^4 conidia/ml for rice infections using the dwarf Indica rice cultivar, CO-39. Conidia were spray-inoculated using an artist's airbrush (Badger, Franklin Park, IL) onto 14-day-old (2–3 leaf stage) plants. Penetration of onion epidermal strips was assessed by the procedure of Chida and Sisler (1987). Incipient cytorrhysis of appressoria was assessed by determining the percentage of appressoria that collapsed after exposure to a range of glycerol concentrations from 0.5 to 5 M (de Jong *et al*., 1997). For each concentration, three replicates of 100 conidia were examined.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

The authors thank Mark Jones for microscopy. This work was supported by a grant to N.J.T. from the Biological Sciences and Biotechnology Research Council (P17477) and studentships to support A.J.F. and J.M.J.

References

Arguelles,J.C. (2000) Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. *Arch. Microbiol.*, **174**, 217–224.

Bell,W., Sun,W.N., Hohmann,S., Wera,S., Reinders,A., De Virgilio,C., Wiemken,A. and Thevelein,J.M. (1998) Composition and functional analysis of the *Saccharomyces cerevisiae* trehalose synthase complex. *J. Biol. Chem.*, **273**, 33311–33319.

Carroll,A.M., Sweigard,J.A. and Valent,B. (1994) Improved vectors for selecting resistance to hygromycin. *Fung. Genet. Newslett.*, **41**, 22.

Chida,T. and Sisler,H.D. (1987) Restoration of appresorial penetration ability by melanin precursors in *Pyricularia oryzae* treated with antipenetrants and in melanin-deficient mutants. *J. Pesticide Sci.*, **12**, 49–55.

Chiu,W.L., Niwa,Y., Zeng,W., Hirano,T., Kobayashi,H. and Sheen,J. (1996) Engineered GFP as a vital reporter in plants. *Curr. Biol.*, **6**, 325–330.

Craig,N.L. (1996) Transposon Tn*7*. *Curr. Top. Microbiol.*, **204**, 27–48.

d'Enfert,C. and Fontaine,T. (1997) Molecular characterization of the *Aspergillus nidulans treA* gene encoding an acid trehalase required for growth on trehalose. *Mol. Microbiol.*, **24**, 203–216.

d'Enfert,C., Bonini,B.M., Zapella,P.D.A., Fontaine,T., da Silva,A.M. and Terenzi,H.F. (1999) Neutral trehalases catalyse intracellular trehalose breakdown in the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. *Mol. Microbiol.*, **32**, 471–483.

de Jong,J.C., McCormack,B.J., Smirnoff,N. and Talbot,N.J. (1997) Glycerol generates turgor in rice blast. *Nature*, **389**, 244–245.

Destruelle,M., Holzer,H. and Klionsky,D.J. (1995) Isolation and characterization of a novel yeast gene, *ATH1*, that is required for vacuolar acid trehalase activity. *Yeast*, **11**, 1015–1025.

De Virgilio,C., Hottiger,T., Dominguez,J., Boller,T. and Wiemken,A. (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast. I. Genetic evidence that trehalose is a thermoprotectant. *Eur. J. Biochem.*, **219**, 1–2, 179–186.

Dixon,K.P., Xu,J-R., Smirnoff,N. and Talbot,N.J. (1999) Independent signalling pathways regulate cellular turgor during hyperosmotic stress and appressorium-mediated plant infection by *Magnaporthe grisea*. *Plant Cell*, **10**, 2045–2058.

Felsenstein,J. (1989) PHYLIP (Phylogeny Interface Package) version 3.2. *Cladistics*, **5**, 164–166.

Fillinger,S., Chaveroche,M.K., van Dijck,P., de Vries,R., Ruijter,G., Thevelein,J. and d'Enfert,C. (2001) Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungi *Aspergillus nidulans*. *Microbiology*, **147**, 1851–1862.

Hamer,L. *et al*. (2001) Gene discovery and gene function assignment in filamentous fungi. *Proc. Natl Acad. Sci. USA*, **98**, 5110–5115.

Hottiger,T., De Virgilio,C., Hall,M.N., Boller,T. and Wiemken,A. (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast II. Physiological concentrations of trehalose increase the thermal stability of proteins *in vitro*. *Eur. J. Biochem.*, **219**, 187–193.

Howard,R.J., Ferrari,M.A., Roach,D.H. and Money,N.P. (1991) Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proc. Natl Acad. Sci. USA*, **88**, 11281–11284.

Kershaw,M.J., Wakley,G. and Talbot,N.J. (1998) Complementation of the *Mpg1* mutant phenotype in *Magnaporthe grisea* reveals functional relationships between fungal hydrophobins. *EMBO J.*, **17**, 3838–3849.

Kopp,M., Müller,H. and Holzer,H. (1993) Molecular analysis of the neutral trehalase gene from *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **268**, 4766–4774.

Londesborough,J. and Varimo,K. (1984) Characterisation of two trehalases in bakers yeast. *Biochem. J.*, **219**, 511–518.

Mittenbühler,K. and Holzer,H. (1988) Purification and characterisation of acid trehalase from the yeast *Suc2* mutant. *J. Biol. Chem.*, **263**, 8537–8543.

Nwaka,S. and Holzer,H. (1998) Molecular biology of trehalose and trehalases in the yeast *Saccharomyces cerevisiae*. *Prog. Nucleic Acid Res. Mol. Biol.*, **58**, 197–237.

Nwaka,S., Kopp,M. and Holzer,H. (1995) Expression and function of the trehalase genes *NTH1* and *YBR0106* in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **270**, 10193–10198.

Page,R.D.M. (1996) TreeView: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.*, **12**, 357–358.

Punt,P.J., Oliver,R.P., Dingemanse,M.A., Pouwels,P.H. and Van den Hondel,C.A.M.J.J. (1987) Transformation of *Aspergillus* based on the hygromycin-B resistance marker from *Escherichia coli*. *Gene*, **56**, 117–124.

Saitou,N. and Nei,M. (1987) The neighbor-joining method—a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406–425.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schuller,C., Brewster,J.L., Alexander,M.R., Gustin,M.C. and Ruis,H. (1994) The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae CTT1* gene. *EMBO J.*, **13**, 4382–4389.

Sweigard,J.A., Carroll,A.M., Farrall,L., Chumley,F.G. and Valent,B. (1998) *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. *Mol. Plant Microbe Interact.*, **11**, 404–412.

Talbot,N.J., Ebbole,D.J. and Hamer,J.E., (1993) Identification and characterisation of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *Plant Cell*, **5**, 1575–1590.

Talbot,N.J. and Foster,A.J. (2001) Genetics and genomics of the rice blast fungus *Magnaporthe grisea*: developing an experimental model for understanding fungal diseases of cereals. *Adv. Bot. Res.*, **34**, 263–287.

Thevelein,J.M. (1984) Regulation of trehalose mobilisation in fungi. *Microbiol. Rev.*, **48**, 42–59.

Thevelein,J.M. (1996) Regulation of trehalose metabolism and its relevance to cell growth and function. In Brambl,R. and Marzluf,G.A. (eds), *The Mycota. III. Biochemistry and Molecular Biology*. Springer Verlag, Berlin, Germany, pp. 395–420.

Thevelein,J.M. and Hohmann,S. (1995) Trehalose synthase: guard to the gate of glycolysis in yeast? *Trends Biochem. Sci.*, **20**, 3–10.

Thines,E., Weber,R.W.S. and Talbot,N.J. (2000) MAP kinase and protein kinase A-dependent mobilisation of triacylglycerol and glycogen during appressorium turgor generation by *Magnaporthe grisea. Plant Cell*, **12**, 1703–1718.

Thompson,J.D., Higgins,D.G. and Gibson,T.J. (1994) CLUSTAL_W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**, 4673–4680.

Timberlake,W.E. (1993) Translational triggering and feedback fixation in the control of fungal development. *Plant Cell*, **5**, 1453–1460.

Tucker,S.L. and Talbot,N.J. (2001) Surface attachment and pre-penetration stage development by plant pathogenic fungi. *Annu. Rev. Phytopathol.*, **39**, 385–417.

Vuorio,O.E., Kalkkinen,N. and Londesborough,J. (1993) Cloning of two related genes encoding the 56-kDa and 123-kDa subunits of trehalose synthase from the yeast *Saccharomyces cerevisiae*. *Eur. J. Biochem.*, **216**, 849–861.

Zaragoza,O., Blasquez,M.A. and Gancedo,C. (1998) Disruption of the *Candida albicans TPS1* gene encoding trehalose-6-phosphate synthase impairs formation of hyphae and decreases infectivity. *J. Bacteriol.*, **180**, 3809–3815.

Zhu,H., Blackmon,B.P., Sasinowski,M. and Dean,R.A. (1999) Physical map and organisation of chromosome 7 in the rice blast fungus, *Magnaporthe grisea*. *Genome Res.*, **9**, 739–750.

Received May 27, 2002; revised November 12, 2002; accepted November 14, 2002