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Tps1 regulates the pentose phosphate pathway, nitrogen metabolism and fungal virulence

Richard A Wilson, Joanna M Jenkinson, Robert P Gibson, Jennifer A Littlechild, Zheng-Yi Wang[1] and Nicholas J Talbot*

School of Biosciences, University of Exeter, Exeter, UK

Trehalose fulfils a wide variety of functions in cells, acting as a stress protectant, storage carbohydrate and compatible solute. Recent evidence, however, indicates that trehalose metabolism may exert important regulatory roles in the development of multicellular eukaryotes. Here, we show that in the plant pathogenic fungus *Magnaporthe grisea* trehalose-6-phosphate (T6P) synthase (Tps1) is responsible for regulating the pentose phosphate pathway, intracellular levels of NADPH and fungal virulence. Tps1 integrates glucose-6-phosphate (G6P) metabolism with nitrogen source utilisation, and thereby regulates the activity of nitrate reductase. Activity of Tps1 requires an associated regulator protein Tps3, which is also necessary for pathogenicity. Tps1 controls expression of the nitrogen metabolite repressor gene, *NMR1*, and is required for expression of virulence-associated genes. Functional analysis of Tps1 indicates that its regulatory functions are associated with binding of G6P, but independent of Tps1 catalytic activity. Taken together, these results demonstrate that Tps1 is a central regulator for integration of carbon and nitrogen metabolism, and plays a pivotal role in the establishment of plant disease.

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Introduction

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a non-reducing sugar that is among the most widespread disaccharides in living cells, occurring in bacteria, plants, insects, some invertebrates and fungi. Trehalose has been implicated in the cellular response to numerous environmental stresses, such as heat-shock, starvation, hyperosmotic shock and desiccation, but it is becoming increasingly clear that trehalose and its immediate precursor, trehalose-6-phosphate (T6P), also regulate growth and development. The absence of T6P synthase (Tps1) in *Arabidopsis thaliana*, for example, causes embryo lethality (Eastmond *et al*, 2002; Gómez *et al*, 2006), while T6P phosphatase is required for inflorescence development in maize (Satoh-Nagasawa *et al*, 2006). The sites of action of T6P and trehalose in the regulation of growth and development in multicellular eukaryotes, however, are largely unresolved.

Recently, we discovered that Tps1 is essential for establishment of rice blast disease by the filamentous fungus *Magnaporthe grisea* (Foster *et al*, 2003). Rice blast is a devastating problem in cultivated rice (for a review see Talbot, 2003) and the fungus infects host plants using specialised infection structures called appressoria, which develop on the rice leaf surface (Veneault-Fourrey *et al*, 2006). T6P synthase was found to be essential for development of functional appressoria and *M. grisea* Δ*tps1* mutants are non-pathogenic (Foster *et al*, 2003). In addition, Δ*tps1* mutants sporulated very poorly, suggesting that Tps1 may play a role in two distinct cellular differentiation events in *M. grisea*—conidiogenesis and appressorium formation (Foster *et al*, 2003).

Loss of Tps1 in yeasts has previously been shown to have severe effects on sugar metabolism. Deletion of the homologous *TPS1* gene in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, for instance, prevents their growth in the presence of glucose. This is associated with overactive hexokinase activity, an increased flux of glucose into glycolysis, irreversible and catastrophic depletion of ATP and free inorganic phosphate (P_i), and accumulation of hexose phosphate intermediates (Luyten *et al*, 1993; Winderickx *et al*, 1996; Ernandes *et al*, 1998; Arguelles, 2000; Gancedo and Flores, 2004). In these yeasts, T6P inhibits hexokinase activity and therefore acts as a means of regulating the entry of glucose into glycolysis. Consequently, glucose-containing media are toxic for *tps1*Δ mutants. In *S. cerevisiae tps1*Δ mutants, deletion of *HXK2*, which encodes hexokinase II, remediates the growth defect on glucose, presumably by slowing entry of glucose into glycolysis (Hohmann *et al*, 1993). Furthermore Tps1, which is part of a multi-enzyme complex with three additional proteins, Tps2, Tps3 and Tsl1 (Reinders *et al*, 1997; Voit, 2003; Elbein *et al*, 2003), may act directly on hexokinase, modulating its activity (Bonini *et al*, 2003). We previously noted that in addition to the developmental phenotypes associated with loss of Tps1 in *M. grisea*, Δ*tps1* mutants were also unable to grow on glucose, but unlike the yeast species their growth could be restored by addition of free amino acids to the medium, suggesting that cross-talk between sugar signalling and nitrogen metabolism occurs in the fungus (Foster *et al*, 2003).

In this study, we set out to determine the role of trehalose biosynthesis in plant infection by *M. grisea*. We decided to explore, in particular, the relationship between the role of Tps1 in sugar metabolism, and its requirement for fungal pathogenicity. Here, we present evidence to show that the

*Corresponding author. School of Biosciences, University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter EX4 4QD, UK.
Tel.: +44 1392 264673; Fax: +44 1392 264668;
E-mail: N.J.Talbot@exeter.ac.uk
[1]Present address: Biotechnology Institute, Zhejiang University, Hangzhou 310029, China

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apparent inability of *M. grisea* Δ*tps1* to grow on glucose as a sole carbon source is not due to glycolytic misregulation, as predicted by studies in yeast, but is instead due to a role for Tps1 in the control of NADPH levels, via regulation of the oxidative pentose phosphate pathway. Furthermore, Tps1 regulates nitrogen utilisation by controlling *NMR1* gene expression and nitrate reductase activity. We also establish that the role of Tps1 in fungal pathogenicity results from regulatory functions of the protein and its control of virulence gene expression, rather than its catalytic function. Taken together, our results demonstrate that the Tps1 protein is a central regulator of infection-related processes in a plant pathogenic fungus.

Results

M. grisea TPS1 restores glycolytic regulation to a S. cerevisiae tps1Δ mutant

In order to investigate the biological function of Tps1 in *M. grisea*, we first investigated the functional relationship between *M. grisea* Tps1 and *S. cerevisiae* Tps1, where a role in the regulation of glycolysis has been clearly established (Hohmann *et al*, 1993). The *M. grisea TPS1* gene was expressed in *S. cerevisiae tps1*Δ::*ura3* mutant MB062 under control of the galactose-inducible *GAL1* promoter. Transformants were grown on growth medium containing galactose and then replica plated onto medium with glucose as sole carbon source (Figure 1A). All transformed strains, either with or without a functional *TPS1* gene, were able to grow on galactose, but only the wild-type W303 strain and the *tps1*Δ::*ura3*:*GAL1*(p):*MgTPS1*:*URA3* strain expressing *M. grisea* Tps1 could grow on glucose, when transferred by replica plating. We conclude that expression of *M. grisea TPS1* is sufficient to restore glycolytic regulation to a yeast *tps1*Δ mutant.

Characterisation of the relationship between Tps1 and the regulation of glycolysis in M. grisea

*S. cerevisiae tps1*Δ mutants accumulate early glycolytic sugar phosphate intermediates when grown with glucose as carbon source, which results from stalled glycolysis (Gancedo and Flores, 2004). We therefore carried out high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) to measure sugar phosphate levels in mycelium of a wild-type *M. grisea* strain Guy-11 and the isogenic Δ*tps1* mutant (Foster *et al*, 2003), as shown in Figure 1B. Significantly higher amounts of G6P ($P \leqslant 0.01$) and fructose-6-phosphate ($P \leqslant 0.01$) were observed when *M. grisea* Δ*tps1* mutants were incubated in glucose minimal medium (GMM), compared to the wild-type strain Guy-11, consistent with misregulation of glycolysis. However, unlike in yeast, fructose-1,6-bisphosphate levels were not elevated in Δ*tps1* strains (Supplementary Figure 1A, available online). To investigate whether control of glycolysis is altered in Δ*tps1* mutants in the same way as in yeast (Hohmann *et al*, 1993), we used bioinformatics to identify the sole hexokinase gene, *HXK1*, of *M. grisea* (Dean *et al*, 2005) and disrupt its function by targeted gene replacement, both in Guy11 and in a Δ*tps1* mutant background. To study the effect of deleting *HXK1* in *M. grisea*, we grew Guy-11, Δ*tps1*, Δ*hxk1* and Δ*tps1* Δ*hxk1* double mutants in CM for 48 h, followed by growth in GMM, with nitrate as nitrogen source, for 16 h. Hexokinase activity

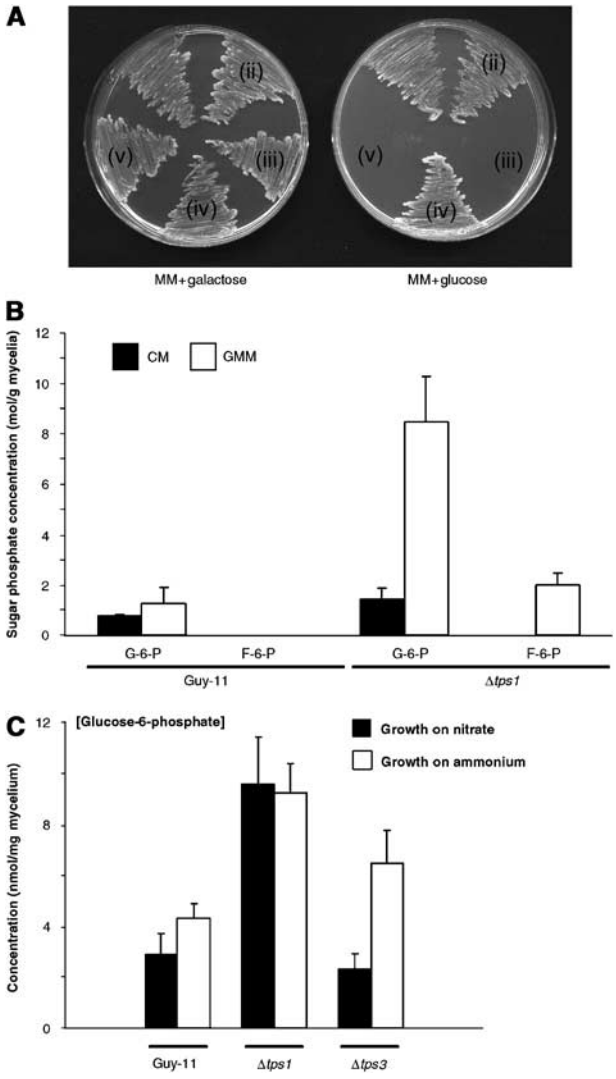


Figure 1 Expression of *M. grisea TPS1* in *S. cerevisiae* and analysis of sugar phosphate metabolism. (**A**) Complementation of *S. cerevisiae tps1*Δ strain MB062 with *MgTPS1*. Yeast strains were plated onto minimal media (MM) with galactose as sole carbon, and compared to growth on glucose yeast medium (GMM). (i, ii) *tps1*Δ::*ura3*:*GAL1*(p):*MgTPS1*:*URA3* (yeast *tps1*Δ deletion strain transformed with *MgTPS1*); (iii) *tps1*Δ::*ura3*:*GAL1*(p): *URA3* (yeast *tps1*Δ deletion strain transformed with empty vector); (iv) W303 (isogenic strain of *S. cerevisiae* with functional *TPS1* gene); (v) *tps1*Δ::*ura3* (*tps1*Δ deletion strain MB062). (**B**) Sugar phosphate accumulation in mycelium of *M. grisea* Guy-11 and Δ*tps1*. Mycelium was grown in CM for 48 h before transferring to CM (closed bars) or GMM (open bars) for 24 h. G6P (G-6-P) and fructose-6-phosphate (F-6-P) levels were determined by HPAE-PAD using a PA100 column (Dionex). Error bars represent s.d. of three replications of the experiment.

was assayed from all four strains and was reduced in Δ*hxk1* and Δ*hxk1* Δ*tps1* mutants (Figure 2A). Consistent with this, the levels of G6P were reduced four-fold in the Δ*hxk1* and Δ*hxk1* Δ*tps1* mutants compared to Guy-11, and reduced almost 14-fold compared to Δ*tps1* strains (Figure 2B). The residual enzyme activity observed in our assay (Figure 2A) in the mutants is likely due to G6P production by glucokinase, although this does not appear to play a major role in G6P synthesis in *M. grisea* (Figure 2B). Trehalose levels were also reduced in Δ*hxk1* mutants, which may result from reduction in the Tps1 substrate G6P (Figure 2C). The ability of *M. grisea*

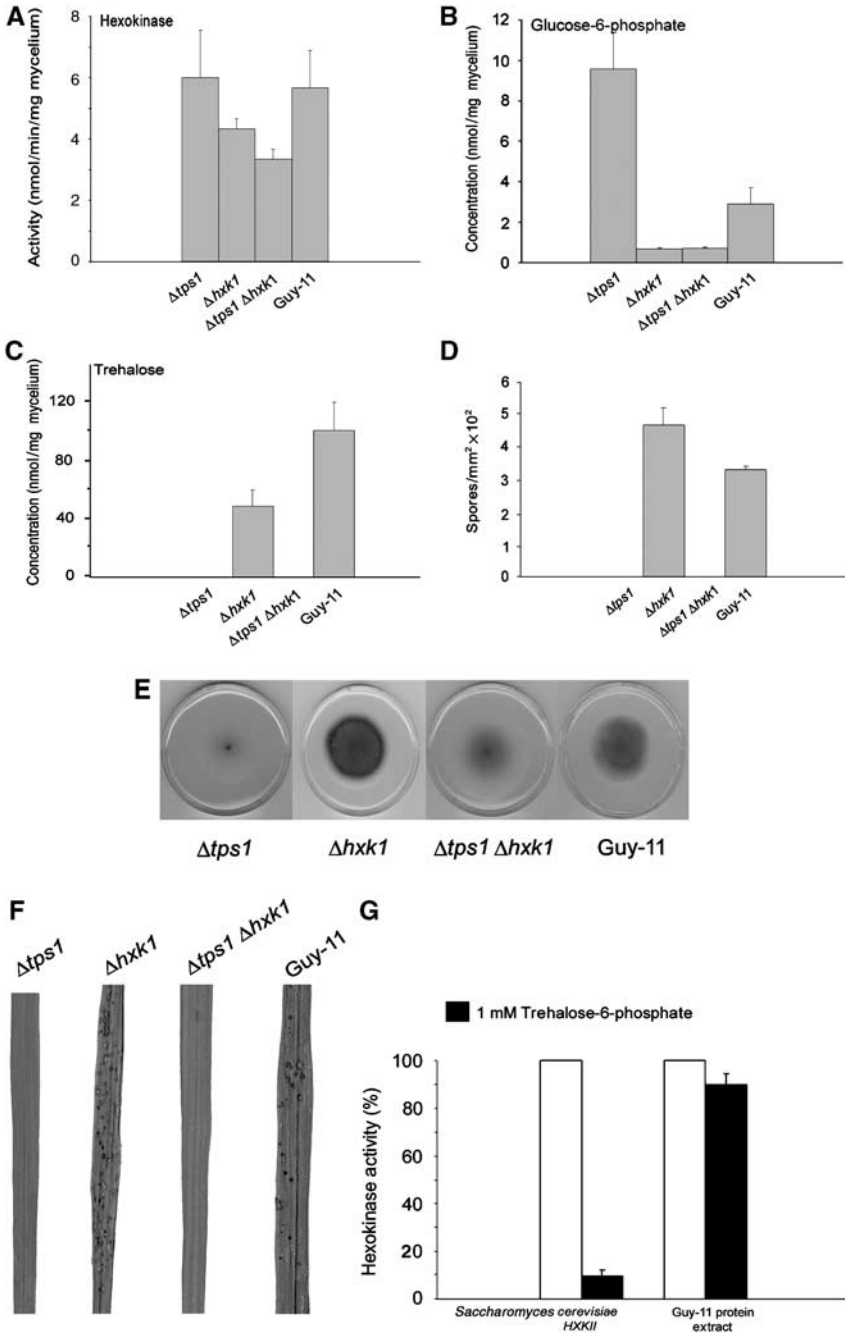


Figure 2 Characterisation of *M. grisea* Δ*hxk1* mutants. (**A**) Hexokinase activity was measured and compared in Δ*tps1*, Δ*hxk1*, Δ*tps1* Δ*hxk1* and Guy-11 strains. Strains were grown in complete medium (CM) for 48 h followed by growth in glucose minimal medium (GMM) for 16 h. (**B**) G6P levels in the same strains and the same conditions. Bars represent standard error of three independent analyses. (**C**) Trehalose levels in the same strains and conditions. Bars represent standard error of three independent analyses. (**D**) Effect of Δ*hxk1* mutation on sporulation. Δ*tps1*, Δ*hxk1*, Δ*tps1* Δ*hxk1* and Guy-11 strains were grown on GMM plates ($n = 4$) for 10 days, followed by harvesting of the spores for counting. Bars represent standard error. (**E**) Effect of Δ*hxk1* mutation on vegetative growth. Δ*tps1*, Δ*hxk1*, Δ*tps1* Δ*hxk1* and Guy-11 strains were grown on GMM plates for 10 days. (**F**) Virulence of Δ*tps1*, Δ*hxk1*, Δ*tps1* Δ*hxk1* and Guy-11 strains on rice seedlings. Rice seedlings were inoculated at a concentration of 5×10^4 spores/ml. (**G**) Hexokinase inhibition by 1 mM T6P. Bars represent standard error of three independent analyses.

Δ*tps1* Δ*hxk1* mutants to grow in the presence of glucose was partially restored compared to Δ*tps1* mutants (Figure 2E), but there was no restoration of sporulation (Figure 2D), or their ability to cause rice blast disease (Figure 2F). Furthermore in *S. cerevisiae* T6P is a potent inhibitor of *HXK2* activity (Gancedo and Flores, 2004), whereas hexokinase activity in a Guy-11 extract is not significantly affected by the presence of T6P (Figure 2G). Interestingly, *S. cerevisiae tps1*Δ diploids do show a sporulation defect that is not repressible by deletion of Hxk2 (Neves *et al*, 1995), suggesting that some effects of this mutation are independent of its role in glycolytic regulation. When considered together, our results suggested that, unlike in yeast, *M. grisea* Δ*tps1* growth phenotypes cannot be remediated by deletion of *HXK1* and that Tps1 may serve distinct functions in sugar metabolism, asexual development and the control of fungal virulence.

M. grisea Δ*tps1 mutants are unable to utilise nitrate as a nitrogen source*

Previously, it was demonstrated that addition of a rich source of amino acids would restore the ability of Δ*tps1* mutants to grow in the presence of glucose (Foster *et al*, 2003). We were intrigued by this observation and therefore grew Δ*tps1* mutants on both GMM supplemented with single amino acids or alternative nitrogen sources (Supplementary Table 1). Δ*tps1* mutants could grow well on GMM with single amino acids (except cysteine, see below) and also grew normally on GMM with ammonium (NH_4^+) or nitrite (NO_2^-), but not nitrate (NO_3^-), as sole nitrogen source irrespective of carbon source (Figure 3A). Thus, the original suggestion that Δ*tps1* mutants cannot grow on glucose (Foster *et al*, 2003) is actually a conditional response mediated by the nature of the available nitrogen source. Because the metabolism of NO_2^- differs from that of NO_3^- by a single enzymatic step, nitrate reductase, we reasoned that *M. grisea* Δ*tps1* mutants could be nitrate non-utilising due to the absence, or suppression, of nitrate reductase. To test this idea, we assayed nitrate reductase activity in Δ*tps1* mutants grown in NO_3^- and ammonium NH_4^+-containing medium following a switch from CM. Guy-11 showed increased nitrate reductase activity after growth in NO_3^- compared to growth on NH_4^+, while under both growth conditions, Δ*tps1* mutants showed almost undetectable levels of nitrate reductase activity (Figure 4A). Consistent with this, Δ*tps1* mutants were resistant to toxic concentrations of potassium chlorate (750 mM) when grown under de-repressing conditions for nitrogen source utilisation (Cove, 1976), confirming the absence of functional nitrate reductase activity in Δ*tps1* (Figure 3B). Therefore, we conclude that the inability of *M. grisea* Δ*tps1* mutants to grow on GMM is not due to glycolytic misregulation, but is instead due to NO_3^- non-utilisation. Supplementation with individual amino acids, ammonium or nitrite thus restores growth by providing an alternative nitrogen source to the fungus.

The role of NADPH in nitrate utilisation

The role of Tps1 in NO_3^- utilisation was unexpected, and we therefore set out to determine why a trehalose biosynthetic

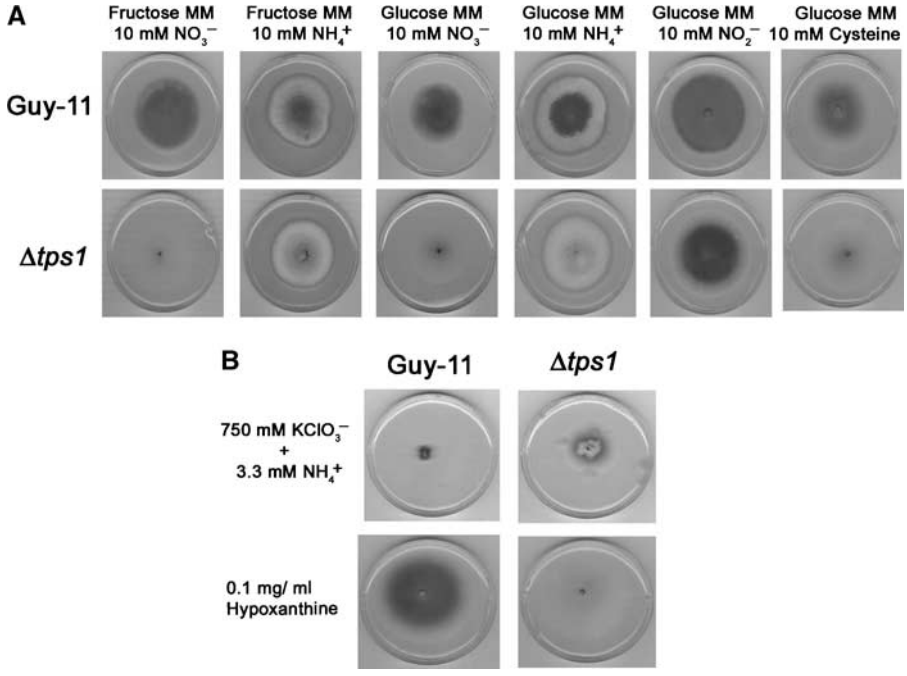
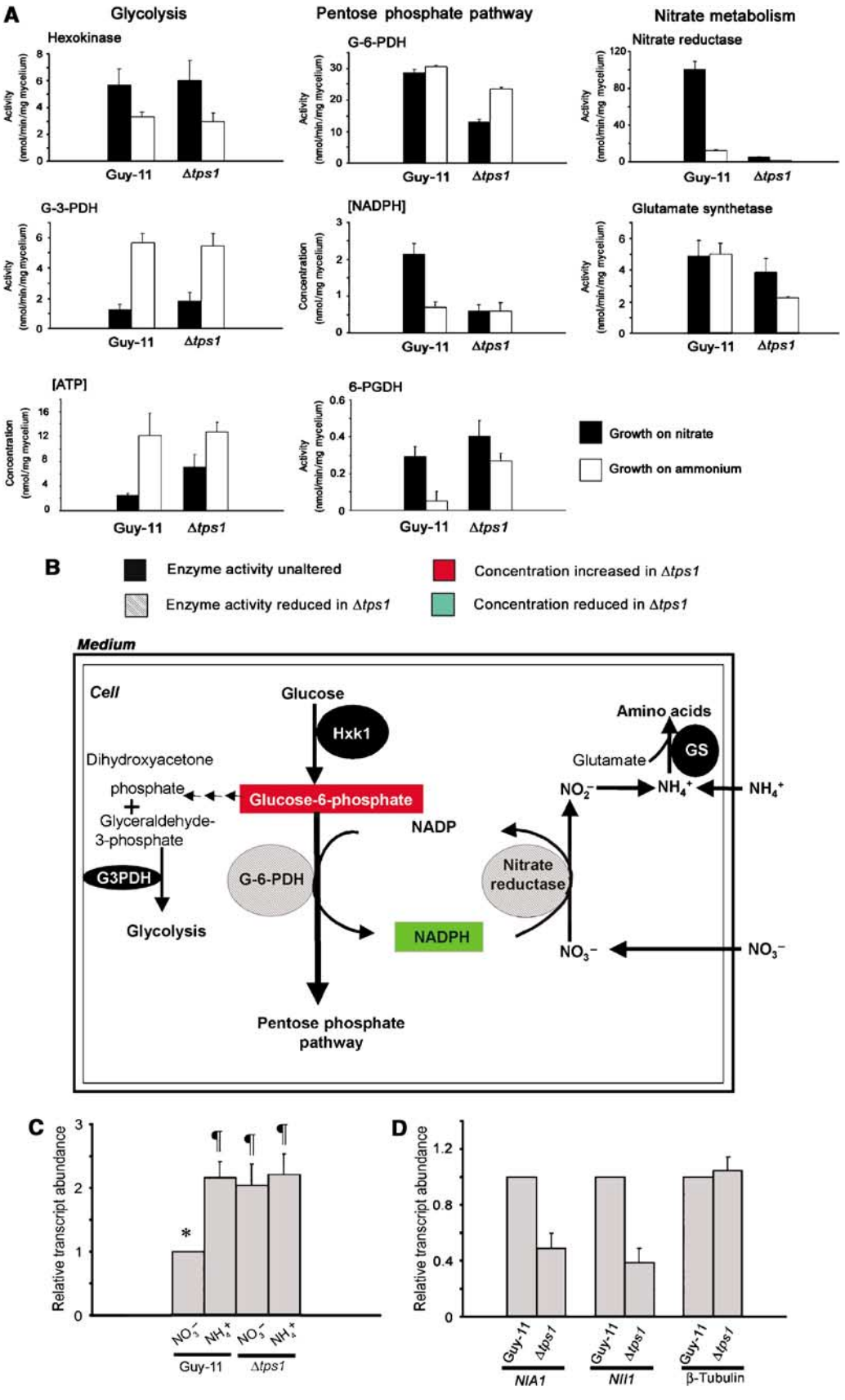


Figure 3 Nitrogen source utilisation and chlorate resistance of Δ*tps1* mutants of *M. grisea*. (**A**) Guy-11 and Δ*tps1* mutants were grown on MM containing 1% fructose or glucose as sole carbon sources. Sole nitrogen sources were provided as indicated, at 10 mM concentrations. *M. grisea* cultures were grown for 10 days at 26°C. (**B**) *M. grisea* strains were grown on GMM supplemented with 750 mM potassium chlorate and 3.3 mM NH_4^+ for 10 days at 26°C. Potassium chlorate is toxic to strains carrying active nitrate reductase enzymes under nitrogen de-repressing conditions (Cove, 1976). Strains were also grown on GMM with 0.1 mg/ml hypoxanthine as sole nitrogen source for 10 days at 26°C.

Figure 4 Carbon and nitrogen metabolism is linked via G6P metabolism and NADPH production. (**A**) Enzyme activities associated with glycolysis, the pentose phosphate pathway and NO_3^- metabolism from Guy-11 and Δ*tps1* strains grown in CM for 48 h followed by incubation in GMM with NO_3^- (closed bars) or NH_4^+ (open bars) for 16 h. Bars represent standard error of three independent replicates. G-3-PDH is glyceraldehyde-3-phosphate dehydrogenase. G-6-PDH is G6P dehydrogenase. 6-PGDH is 6-phosphogluconic dehydrogenase. (**B**) Schematic representation of the effect of Δ*tps1* mutation on carbon and nitrogen metabolism in *M. grisea*. Differences in metabolite accumulation and enzyme activity between Guy-11 and Δ*tps1* strains are shown. (**C**) Transcript analysis of *NMR1*. Strains were grown for 48 h in CM followed by incubation in GMM with NO_3^- or NH_4^+ as a sole nitrogen source for an additional 16 h. Total RNA was extracted and gene expression analysed by RT–PCR. Analysis was performed in triplicate and normalised using levels of actin gene expression. Transcript abundance was calculated relative to *NMR1* expression in Guy-11 on GMM with nitrate. Values with a different letter above are significantly different (Student's *t*-test, $P<0.05$). (**D**) Transcript analysis of *NIA1*, *NII1* and β-tubulin encoding gene. Strains were grown for 48 h in CM followed by incubation in GMM with NO_3^- as a sole nitrogen source for an additional 16 h. Total RNA was extracted and gene expression analysed by RT–PCR. Analysis was performed in triplicate and normalised using levels of actin gene expression. Transcript abundance was calculated relative to gene expression in Guy-11 on GMM with NO_3^-.

enzyme should be necessary for nitrate reductase activity. In filamentous fungi, NO_3^- is metabolised to NO_2^- by the action of nitrate reductase, followed by reduction of NO_2^- to NH_4^+ by nitrite reductase (Cove, 1966). Ammonium is assimilated into amino acids through the action of glutamine synthetase and the GOGAT pathway (Kusnan *et al*, 1987). Nitrate reductase



requires NADPH as a cofactor for function, and NADPH is generated from G6P by the action of G6P dehydrogenase (G-6-PDH) in the oxidative pentose phosphate pathway. G6P is, as described previously, also a substrate of Tps1 (Bell *et al*, 1998). In *Aspergillus nidulans*, transfer from growth on NH_4^+ to growth on NO_3^- leads to increased nitrate reductase and nitrite reductase activities, and also stimulates hexokinase and the enzymes of the pentose phosphate pathway, thereby increasing NADPH production from G6P (Hankinson and Cove, 1974). We therefore assayed the activities of enzymes involved in synthesis of NADPH and catabolism of NO_3^- in both Guy-11 and Δ*tps1* mutants (Figure 4A). Incubation in NO_3^- led to an increase in hexokinase activity in both strains, suggesting that elevated levels of G6P are required by *M. grisea* under conditions of NO_3^- utilisation (Dunn-Coleman and Pateman, 1979) and, secondly, that the increase in hexokinase activity does not require Tps1. In addition, other enzyme activities of the glycolytic pathway, such as glyceraldehyde-3-phosphate dehydrogenase (Figure 4A), phosphofructokinase and phosphoglucose isomerase (Supplementary Figure 1B) were not affected by the Δ*tps1* mutation, while levels of ATP were similar in both Δ*tps1* and Guy-11 (Figure 4A). These results underline the fact that glycolytic misregulation and ATP depletion does not occur in *M. grisea* Δ*tps1* mutants during growth on glucose-containing media.

In the wild-type *M. grisea* strain Guy-11, a large increase in NADPH levels was observed during growth on NO_3^- (Figure 4A). In contrast, NADPH levels did not increase in Δ*tps1* during growth on NO_3^- and there was also a relative decrease in G-6-PDH activity (Figure 4A) despite the fact that high levels of G6P accumulated in the Δ*tps1* mutant during growth on both nitrogen sources (Supplementary Figure 1C). We did not detect any inhibitory effect of G6P on G-6-PDH activity *in vitro* (Supplementary Figure 1D). Glutamine synthetase levels were similar in Guy-11 and Δ*tps1* strains, suggesting that the GS-GOGAT pathway operates normally in the absence of Tps1. The activity of 6-phosphogluconic dehydrogenase, the next enzyme of the pentose phosphate pathway, is also not reduced in Δ*tps1*. From these data we can conclude that regulation of G6PDH and NADPH production, in response to NO_3^-, is a Tps1-dependent process in *M. grisea* (Figure 4B).

It is worth noting that two NADPH-dependent proteins are required for the metabolism of L-cysteine in eukaryotes. Cysteine oxygenase (EC 1.13.11.20) converts L-cysteine to 3-sulfinoalanine (Lombardini and Singer, 1969) and precedes conversion of 3-sulfinoalanine to alanine, while NADPH-dependant coenzyme A disulfide-glutathione reductase (EC 1.8.1.10; Ondarza *et al*, 1969) is necessary for the reduction of L-cysteine. Interestingly, the only amino acid that Δ*tps1* mutants are unable to utilise as a sole nitrogen source is cysteine (Figure 3A), consistent with the observed deficiency in NADPH.

Genetic regulation of nitrate utilisation genes via Tps1

The absence of nitrate reductase activity in Δ*tps1* mutants during growth on NO_3^- could be due either to an inactive enzyme or to repression of the *NIA1* gene, which encodes nitrate reductase. Adding exogenous NADPH to the nitrate reductase activity enzymatic assay did not result in detectable nitrate reductase activity in Δ*tps1* (Figure 4A), suggesting that there is no nitrate reductase protein present in the cell. We also noted that Δ*tps1* mutants cannot utilise hypoxanthine as a nitrogen source (Figure 3B). Nitrate reductase and xanthine dehydrogenase (used to metabolise hypoxanthine) both require a molybdenum-containing cofactor, encoded in *A. nidulans* by the *cnx* genes (Unkles *et al*, 1999). Expression of *cnx* and *niaD* genes requires a positively acting GATA transcription factor encoded by the *areA* gene in *A. nidulans* (Wilson and Arst, 1998) and the *NUT1* gene in *M. grisea* (Froeliger and Carpenter, 1996). The activity of AreA is negatively regulated by the product of *nmrA* (Andrianopoulos *et al*, 1998). We performed RT–PCR to analyse the gene expression of *NMR1*, the equivalent *M. grisea* gene, in Guy11 and Δ*tps1* during growth on nitrate and ammonium (Figure 4C). Expression of *NMR1* was significantly higher in Δ*tps1* mutants than in Guy-11 during growth on nitrate, and was comparable to expression levels observed during growth of Guy-11 and Δ*tps1* on ammonium when it fully represses the action of the AreA GATA factor (Andrianopoulos *et al*, 1998). Furthermore, high levels of *NMR1* transcript in Δ*tps1* strains corresponded with reduced expression of *NIA1* and *NII1*, encoding nitrate and nitrite reductase respectively, compared to Guy-11 (Figure 4D). In *A. nidulans*, nitrate and nitrite reductase genes are tightly linked and coordinately regulated from a shared promoter (Cove, 1976). However, *M. grisea NIA1* and *NII1* genes are not linked and reside on chromosomes III and VI, respectively (Dean *et al*, 2005). We wondered if, considering Δ*tps1* strains could grow on NO_2^- but not NO_3^- media, there was evidence for differential regulation of nitrate and nitrite reductase activities in *M. grisea*. Interestingly, unlike in *A. nidulans*, we were unable to detect nitrate reductase activity in protein extracts of Guy-11 and Δ*tps1* strains grown in CM followed by a switch to NO_2^--containing media (not shown). In addition, NADPH levels of Guy-11 and Δ*tps1* strains were not elevated during growth on NO_2^- compared to NH_4^+ (Supplementary Figure 2). This suggests that nitrate and nitrite reductase activities are not always coordinately regulated, and second that NADPH may not be important for nitrite reductase activity. Studies of *Neurospora crassa* nitrite reductase (Nason *et al*, 1954) demonstrated that the enzyme can utilise both NADH and NADPH as cofactor *in vitro*, and the preferred cofactor for *M. grisea* NII1 therefore needs to be determined. We conclude that in response to NO_3^-, Tps1 is required to regulate the activity of G6PDH, which controls levels of G6P, NADPH and nitrate reductase activity, and also to modulate gene expression by control of *NMR1*.

Genetic regulation of Tps1 activity in M. grisea by Tps3

The catalytic subunit of Tps1, encoded by *TPS1*, is part of a multi-protein complex in yeast, with the Tps2 T6P phosphohydrolase and two other proteins, Tps3 and Tsl1 (Gancedo and Flores, 2004). To investigate the control of *M. grisea* Tps1 activity, we analysed the genome sequence of *M. grisea* (Dean *et al*, 2005), identified a homologue of *TPS3* and generated a Δ*tps3* mutant. The Δ*tps3* mutant was impaired in both trehalose (see Figure 6D) and T6P synthesis. HPAE-PAD analysis of mycelial extracts showed concentrations of 99.8 ± 8 pmol per milligram T6P mycelium ($n = 3$) in Guy-11, but no detectable T6P in mycelium of Δ*tps3* mutants (not shown). The Δ*tps3* mutant grew better than Δ*tps1* on NO_3^-, but was still reduced in sporulation (Figure 5). NADPH was

also reduced and showed no increase during growth of Δ*tps3* mutants on NO_3^--containing medium, consistent with the Δ*tps1* phenotype. Interestingly, however, nitrate reductase activity in Δ*tps3* mutants was equivalent to that in Guy-11 during growth on NO_3^- and NH_4^+ (Figure 5B). Nitrate reductase activity is assayed by addition of exogenous NADPH, so while this result indicates that nitrate reductase is present in Δ*tps3* mutants, it is unlikely to be active in the cell due to the lack of endogenous cellular NADPH (Figure 5C). Δ*tps3* mutants are, for example, resistant to potassium chlorate under nitrogen de-repressing conditions (Figure 5A). In addition, Δ*tps3* does not accumulate G6P when grown on NO_3^- (Supplementary Figure 1C) or in CM (Supplementary Figure 1E). Taken together, our results indicate that Tps1 and Tps3 are both necessary for T6P synthesis and regulation of the pentose phosphate pathway and NADPH production, while at least Tps1 is also necessary for control of *NMR1* expression. Significantly, Δ*tps3* mutants are also unable to cause rice blast disease (Figure 5D), demonstrating that fungal pathogenicity requires Tps3-dependent Tps1 activity.

Functional analysis of M. grisea Tps1

The cellular pool of G6P links carbon and nitrogen metabolism via the pentose phosphate pathway and NADPH generation (see Figure 4B), and so we reasoned that Tps1 might act as a reporter of G6P levels, leading to increased G6PDH activity, increased NADPH levels and de-repression of NO_3^- utilisation genes in the presence of NO_3^-. To test this idea, we undertook a functional analysis of the Tps1 protein and characterised the G6P binding pocket of Tps1 by site-directed mutagenesis. A crystal structure for the prokaryotic Tps1 homologue, encoded by *otsA* in *Escherichia coli*, has been used to identify amino-acid residues involved in substrate binding (Gibson *et al*, 2002). We generated a model of *M. grisea* Tps1 based on the OtsA structure (Figure 6A), to identify residues in the G6P-binding pocket that are conserved between OtsA and Tps1, and introduced nucleotide changes into a full-length genomic clone of *TPS1* that would result in four amino-acid substitutions at these positions in Tps1 when the alleles were transformed into a *M. grisea* Δ*tps1* mutant (Figure 6B).

The resulting *M. grisea* Δ*tps1*::*Tps1*^{R22G}, Δ*tps1*::*Tps1*^{Y99V}, Δ*tps1*::*Tps1*^{W108S} and Δ*tps1*::*Tps1*^{D153G} strains were all unable to synthesize trehalose (Figure 6D) or T6P (not shown), indicating the importance of each residue for Tps1 activity. Δ*tps1*::*Tps1*^{R22G}, Δ*tps1*::*Tps1*^{W108S} and Δ*tps1*::*Tps1*^{D153G} strains resembled Δ*tps1* mutants, were NO_3^- non-utilising (Figure 6C) and did not sporulate on minimal medium—although nitrate reductase activity was detected in extracts from Δ*tps1*::*Tps1*^{R22G} strains (Supplementary Figure 1F) suggesting that like Δ*tps3*, this mutant has inactive nitrate reductase present in the cell—whereas strains carrying the Y99V substitution (Δ*tps1*::*Tps1*^{Y99V}) produced increased numbers of spores on minimal medium and sporulated almost as well as Guy11 on CM (Figure 6C). G6P levels were not elevated in Δ*tps1*::*Tps1*^{R22G}, Δ*tps1*::*Tps1*^{Y99V} and Δ*tps1*::*Tps1*^{D153G} strains, suggesting that G6P accumulation is related to the absence of Tps1 protein and not to a non-functional Tps1 variant such as Tps1^{D153G} (Supplementary Figure 1E).

Significantly, the expression of *TPS1* alleles carrying R22G and Y99V substitutions restored the ability to cause rice blast disease when expressed in a Δ*tps1* mutant, as shown in Figure 6E. This shows that the catalytic activity of Tps1 is not required for fungal pathogenicity, because Δ*tps1*::*Tps1*^{R22G} and Δ*tps1*::*Tps1*^{Y99V} strains are unable to synthesize trehalose and T6P, but can still cause disease in rice. In a converse experiment, the *S. cerevisiae TPS1* homo-

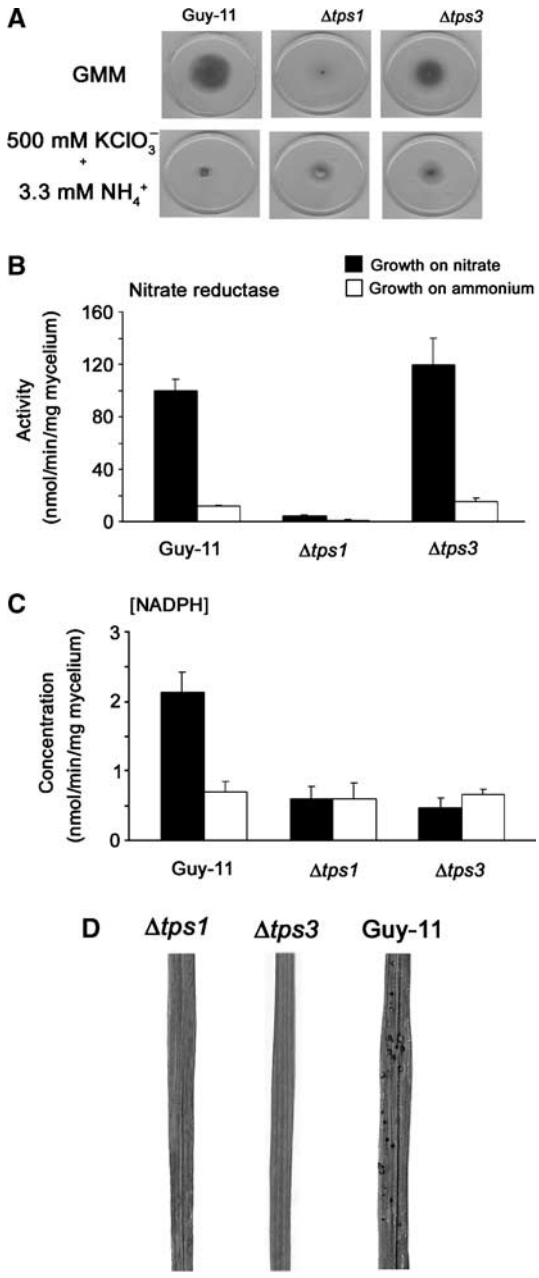


Figure 5 Genetic control of Tps1 activity by Tps3. (**A**) Guy-11, Δ*tps1* and Δ*tps3* strains were grown on GMM and GMM supplemented with 500 mM potassium chlorate and de-repressing concentrations (3.3 mM) of NH_4^+. (**B**) Nitrate reductase activity in Guy-11, Δ*tps1* and Δ*tps3* strains following 48 h growth in CM and 16 h growth in GMM with NO_3^- or NH_4^+. Activities and concentrations were measured in triplicate. Bars represent standard error. (**C**) NADPH concentration in Guy-11, Δ*tps1* and Δ*tps3* strains following 48 h growth in CM and 16 h growth in GMM. Activities and concentrations were measured in triplicate. Bars represent standard error. (**D**) Guy-11, Δ*tps1* and Δ*tps3* strains were inoculated onto rice seedlings to analyse pathogenicity. Spores were inoculated at a rate of 5×10^4 spores/ml.

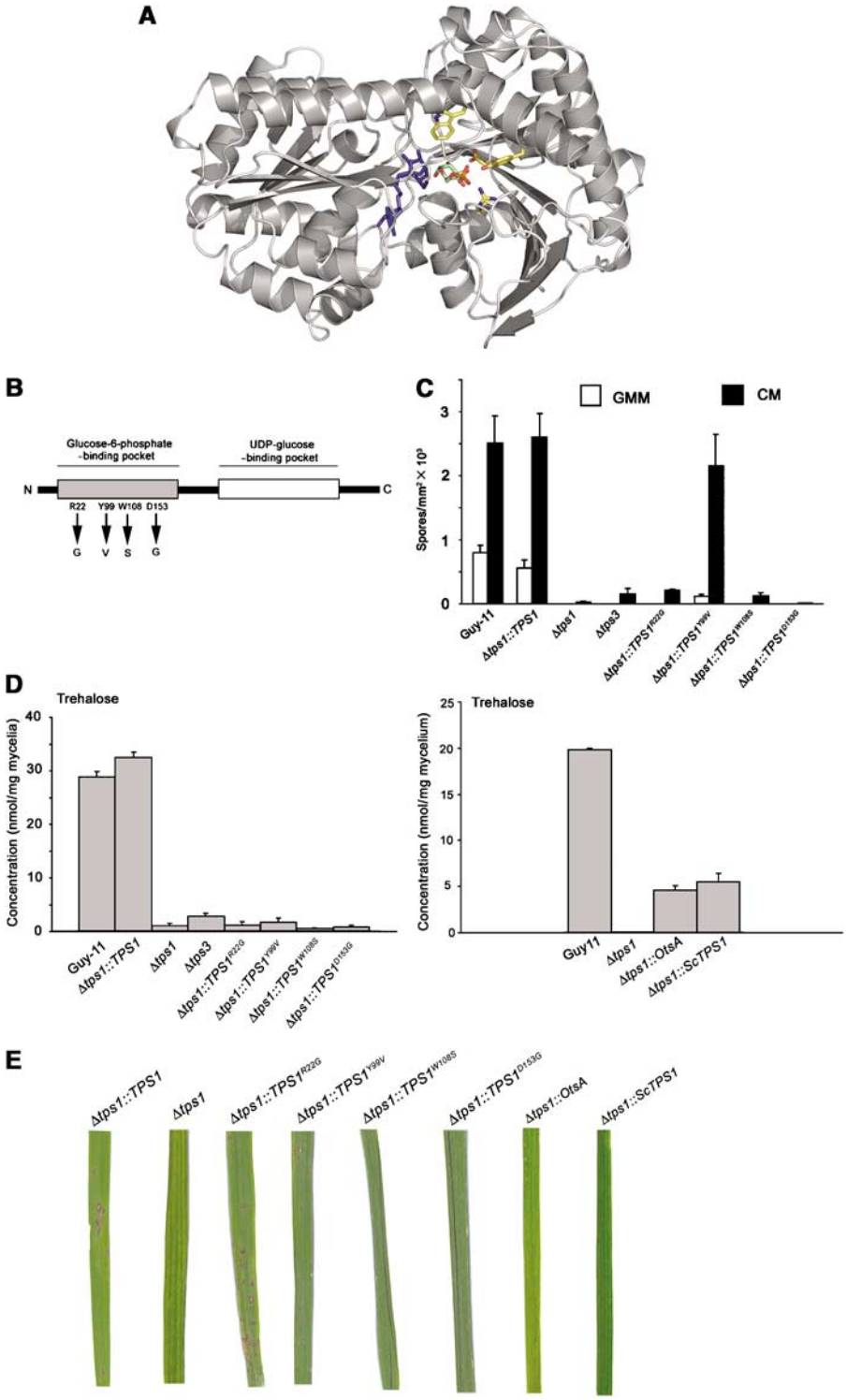


Figure 6 Site-directed mutagenesis of the G6P-binding pocket of Tps1. (**A**) Energy-minimised Tps1 homology model structure constructed from OtsA template structures (Gibson *et al*, 2002) by MODELLER, and showing residues involved in G6P binding. Residues, which were mutated are (clockwise from top) Trp108, Asp153, Tyr99 and Arg22. The locations of G6P (green and red) and UDP-glucose (blue) are based on the template structures to indicate their proximity to the mutated residues. (**B**) Schematic representation of the relative positions of introduced amino-acid substitutions in the G6P-binding pocket of Tps1. The drawing is not to scale. (**C**) Spore production was measured for Guy-11, Δ*tps1*::*TPS1*, Δ*tps1*, Δ*tps3*, Δ*tps1*::*Tps1*^{R22G}, Δ*tps1*::*Tps1*^{Y99V}, Δ*tps1*::*Tps1*^{W108S} and Δ*tps1*::*Tps1*^{D153G} strains. Spores from strains grown for 10 days on CM and GMM were counted from four independent plates. (**D**) Trehalose concentration in Guy-11, Δ*tps1*::*TPS1*, Δ*tps1*, Δ*tps3*, Δ*tps1*::*Tps1*^{R22G}, Δ*tps1*::*Tps1*^{Y99V}, Δ*tps1*::*Tps1*^{W108S} and Δ*tps1*::*Tps1*^{D153G} strains (left bar chart) and Guy-11, Δ*tps1*, Δ*tps1*::*otsA* and Δ*tps1*::*ScTPS1* (right bar chart). Trehalose was measured in triplicate. Bars represent standard error. (**E**) The virulence of Δ*tps1*::*TPS1*, Δ*tps1*, Δ*tps1*::*Tps1*^{R22G}, Δ*tps1*::*Tps1*^{Y99V}, Δ*tps1*::*Tps1*^{W108S} and Δ*tps1*::*Tps1*^{D153G}, Δ*tps1*::*otsA* and Δ*tps1*::*ScTPS1* strains was analysed by inoculating spores onto rice seedlings of cultivar CO-39. Spores were inoculated at a concentration of 5×10^4 spores/ml and rice blast disease symptoms observed after 96 h.

logue *ScTPS1*, and the bacterial homologue *otsA*, were expressed in *M. grisea* Δ*tps1* strains under control of the native *M.* grisea TPS1 promoter. The expression of both genes resulted in elevated trehalose production compared to Δ*tps1* (Figure 6D). However, neither gene complemented the Δ*tps1* mutation for growth on GMM (not shown) or for restoration of virulence (Figure 6E). Therefore, trehalose production in the absence of *M. grisea* Tps1 is not sufficient for virulence.

Analysis of these mutants indicated that NO_3^- utilisation, sporulation, trehalose production and virulence are four independent processes influenced by the Tps1 protein independently of its catalytic activity. At least two residues in the G6P-binding pocket, W108S and D153, are necessary for all of these processes, suggesting that the regulation of fungal virulence and NO_3^- utilisation involves efficient G6P binding to Tps1. Indeed, from our model, we see that amino-acid changes resulting in the greatest perturbation of G6P binding correspond to the greatest loss of Tps1-dependent processes. R22G and Y99V are predicted to reduce the number of contacts made to the phosphate moiety of G6P, and R22G would also eliminate a key interaction promoting closure of the catalytic loop over the active site (Supplementary Figure 3A and B; Gibson *et al*, 2002). However, contacts from other residues remain to position G6P in the active site. W108S and D153G, on the other hand, are predicted to have more profound effects on G6P binding, with both mutations resulting in loss of positioning of the substrate. D153G is predicted to eliminate the only hydroxyl interactions between G6P and the protein (Supplementary Figure 3AII; Gibson *et al*, 2002), thus permitting a larger range of movement for the bound substrate. Similarly, W108S results in loss of the indole moiety necessary for selection of the α-glucose anomer, and this change could allow the more prevalent β-anomer to bind, hence inhibiting Tps1 function (Supplementary Figure 3BV; Gibson *et al*, 2002).

Investigating the role of Tps1 in plant infection-related development

We previously demonstrated that appressoria of Δ*tps1* mutants are reduced in turgor and cannot infect rice leaves (Foster *et al*, 2003). Glycogen is present in the conidium of *M. grisea* and is believed to contribute to cellular turgor as a substrate for glycerol synthesis in the appressorium (de Jong *et al*, 1997; Thines *et al*, 2000). During appressorium development, glycogen accumulation occurs before the onset of turgor generation, and is broken down as glycerol is synthesised (Thines *et al*, 2000). Because glycogen synthesis also requires G6P, we decided to test whether Tps1 is necessary for regulation of glycogen mobilisation in the appressorium. We observed glycogen deposits in conidia of Δ*tps1* mutants, but could not detect glycogen in appressoria, in contrast to Guy11 (Figure 7A). We therefore quantified glycogen in Guy-11 and Δ*tps1* mycelium, grown either on NO_3^- or NH_4^+-containing medium and found that Δ*tps1* mutants accumulate more glycogen when grown in the presence of NO_3^- than NH_4^+, in contrast to Guy11, which accumulates greater glycogen when grown in NH_4^+ (Figure 7B). To investigate the process in more detail, we measured activities of two glycogen biosynthetic enzymes: phosphoglucomutase, which converts G6P to G1P for further conversion to UDP-glucose (Figure 7C), and glycogen synthase, which assembles glycogen from UDP-glucose subunits (Figure 7D). The activities of these two enzymes were slightly reduced in Δ*tps1* compared to Guy-11. Glycogen phosphorylase activity was, however, markedly reduced in Δ*tps1* mutants compared to Guy11 (Figure 7E). Therefore, Tps1 appears to be required for glycogen metabolism in conidia and mycelium, with reduced glycogen phosphorylase activity in Δ*tps1* resulting in glycogen accumulation. The Δ*tps3* mutation also affects glycogen steady-state levels in the conidium (Figure 7A). Interestingly, Δ*tps1*::*Tps1*^{Y99V}, which is unable to synthesize trehalose, but is pathogenic, also affects glycogen steady-state levels in the conidium during appressorium development. We can therefore conclude that inappropriate glycogen accumulation in the conidium is associated with loss of Tps1 in *M. grisea*, but is not sufficient to explain the loss of virulence exhibited by Δ*tps1* mutants. To determine the wider effect of Tps1 on expression of other virulence-associated functions expressed during rice infection, gene expression of the *MPG1* hydrophobin gene and *PTH11*, a G-protein-coupled receptor-encoding gene, both of which are expressed prior to appressorium development (DeZwaan *et al*, 1999; Soanes *et al*, 2002a, b), was analysed. Expression of both *MPG1* and *PTH11* was reduced in the Δ*tps1* mutant compared to Guy-11 (Supplementary Figure 4). We conclude that Tps1 is necessary for expression of at least two major virulence-associated genes during appressorium formation by *M. grisea*.

Discussion

In this study, we set out to determine the role of trehalose and T6P synthesis in the establishment of rice blast disease. The absence of Tps1 from *M. grisea* prevents the fungus from causing disease and also affects spore production, appressorium function and sugar metabolism (Foster *et al*, 2003). Here, we have shown that the importance of Tps1 to cellular differentiation and fungal virulence results from its wide ranging role as a central regulator of both sugar metabolism and nitrogen source utilisation, acting both post-translationally to regulate enzymatic activities, but also as a regulator of gene expression.

The role of Tps1 in yeast has been extensively studied, and in addition to its biosynthetic function, the Tps1 protein controls entry of glucose into glycolysis by regulating the level of G6P, one of the substrates for Tps1. The *M. grisea TPS1* gene has the capacity to fulfil such a function because it is able to complement a yeast *tps1*Δ strain and regulate glycolysis normally. Unlike *M. grisea* Δ*tps1* mutants, however, the growth of yeast *tps1*Δ mutants cannot be restored on glucose-containing medium regardless of the nitrogen source provided, including ammonium (data not shown). It is worth noting, however, that yeast two-hybrid data (Gavin *et al*, 2002) suggests that there is an interaction between *S. cerevisiae* Tps1 and Mks1, a protein involved in regulation of nitrogen utilisation in yeast (Edskes *et al*, 1999).

It became increasingly clear during the course of this study that Tps1 does not regulate glycolysis in *M. grisea* in the same manner as occurs in yeast (Hohmann *et al*, 1993). Deletion of the hexokinase gene *HXK1*, for example, did not remediate growth or sporulation of Δ*tps1* on glucose medium and Δ*tps1* Δ*hxk1* double mutants were still non-pathogenic. Enzyme assays also showed that the activity of glycolytic enzymes was not affected in Δ*tps1* mutants, ATP depletion and fructose 1,6-bisphosphate accumulation did not occur in the

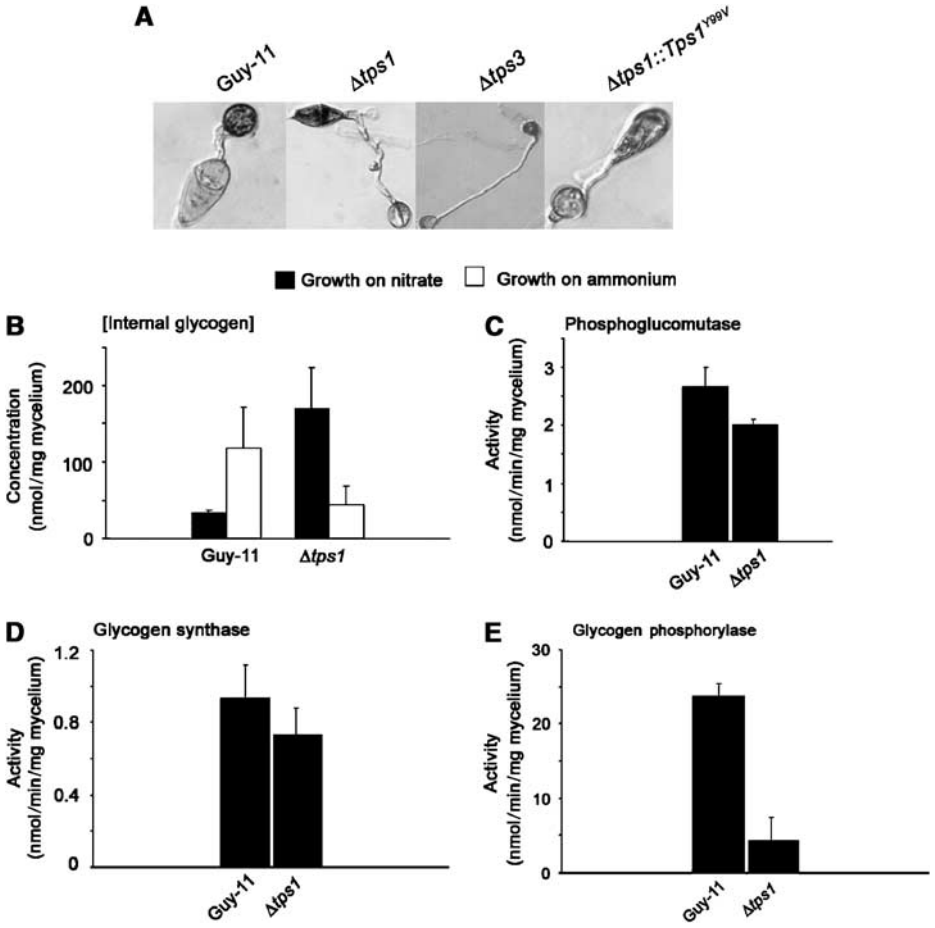


Figure 7 Role of Tps1 in glycogen metabolism and infection-related development. (**A**) At 24 h post-germination, glycogen accumulates in the appressorium of Guy-11 while Δ*tps1*, Δ*tps3* and Δ*tps1*::*Tps1*^{Y99V} strains retain and accumulate glycogen in the conidium. (**B**) Glycogen concentration in Guy-11 and Δ*tps1* strains grown in CM for 48 h followed by growth in GMM with nitrate or ammonium as a sole nitrogen source for 16 h. Measurements were performed in triplicate and bars represent standard error. (**C**) Phosphoglucomutase activities in Guy-11 and Δ*tps1* strains grown in CM for 48 h followed by growth in GMM with NO_3^- as a sole nitrogen source for 16 h. Measurements were performed in triplicate and bars represent standard error. (**D**) Glycogen synthase activities in Guy-11 and Δ*tps1* strains grown in CM for 48 h followed by growth in GMM with NO_3^- as a sole nitrogen source for 16 h. Measurements were performed in triplicate and bars represent standard error. (**E**) Glycogen phosphorylase activities in Guy-11 and Δ*tps1* strains grown in CM for 48 h followed by growth in GMM with NO_3^- as a sole nitrogen source for 16 h. Measurements were performed in triplicate and bars represent standard error.

presence of glucose, and T6P did not inhibit *M. grisea* hexokinase activity. Moreover, expression of *S. cerevisiae TPS1* did not complement a *M. grisea* Δ*tps1* mutant. Growth tests meanwhile demonstrated instead that *M. grisea* Δ*tps1* mutants were NO_3^- non-utilising. We postulated that the connection between NO_3^- utilisation and sugar metabolism in the rice blast fungus was the availability of NADPH, generated from G6P in the oxidative pentose phosphate pathway. Analysis of enzymes involved in generating NADPH showed that in *M. grisea*, as demonstrated previously in *A. nidulans* (Hankinson and Cove, 1974; Dunn-Coleman and Pateman, 1979), hexokinase activity and NADPH levels both increase during growth on NO_3^-, while in Δ*tps1*, G-6-PDH activity and NADPH production are both reduced significantly when the mutant is grown on minimal medium with NO_3^-. Consequently, nitrate reductase levels were not elevated in Δ*tps1* mutants during growth on NO_3^-, explaining why they are unable to grow, and G6P accumulated in cells (Figure 1B). Gene expression analysis, meanwhile, showed that Δ*tps1* mutants express *NMR1*, a negative repressor of nitrogen metabolism, at high levels during incubation in NO_3^--containing medium (Dunn-Coleman *et al*, 1981; Andrianopoulos *et al*, 1998), while expression of the nitrate and nitrite reductase structural genes was reduced in Δ*tps1* strains. This provides evidence that Tps1 is involved in de-repression, via *NMR1*, of genes involved in nitrate utilisation, in addition to its role in stimulation of the pentose phosphate pathway and NADPH production. *A. nidulans* NmrA has been shown to discriminate between oxidised and reduced dinucleotides, including $NADP^+$ and NADPH, and may act as a redox sensor (Lamb *et al*, 2003). The extracellular nitrate signal could act on Tps1 directly via a nitrogen sensing mechanism, or, more likely, via increased G6P levels, due to stimulated hexokinase activity. Additionally, because glycolytic enzyme activities are unaltered in Δ*tps1* compared to Guy-11, and are relatively low in both strains compared to G-6-PDH activity in Guy-11, this suggests the major pathway for glucose consumption in *M. grisea* may be the pentose phosphate pathway. In the absence of *TPS1* therefore, G6P accumulates but G6PDH is not active. We conclude that Tps1 integrates carbon and nitrogen metabolism, probably through G6P sensing, resulting in increased NADPH production

and induction of gene expression associated with nitrate utilisation.

The role of Tps1 in plant disease

To address the role of Tps1 in pathogenicity and to distinguish this from the other biochemical and regulatory functions of the protein, we decided to carry out a structure–function study. Amino-acid substitutions in Tps1 demonstrated that trehalose synthesis is not required for plant infection by *M. grisea*, but that the protein itself, and in particular its efficient binding to G6P, is necessary for its regulatory functions and for its role in fungal virulence. The Δ*tps1*::*Tps1*^{R22G} and Δ*tps1*::*Tps1*^{Y99V} strains, which lack Tps1 catalytic activity, but may allow some G6P binding to Tps1, based on structural modelling of the Tps1 protein with the *E. coli* otsA structure (Gibson *et al*, 2002), are still able to cause rice blast disease in response to G6P binding, even though they are unable to synthesize trehalose. Tps1 therefore acts as an integrator of the carbon and nitrogen status of the cell, perhaps acting as a reporter of G6P levels. Consistent with this, Δ*tps1*::*Tps1*^{W108S} and Δ*tps1*::*Tps1*^{D153G} strains, in which the Tps1 protein is very unlikely to bind to G6P, cannot cause rice blast disease. Additionally, Tps1 can be thought of as being central to a complex network of interactions and cellular processes responding to nitrogen and carbon signals in the cell and resulting in the potential Tps1 interactome depicted in Figure 8. Analysis of Δ*tps1*::*Tps1*^{R22G}, Δ*tps1*::*Tps1*^{W108S}, Δ*tps1*::*Tps1*^{Y99V}, Δ*tps1*::*Tps1*^{D153G} and Δ*tps3* mutants has demonstrated that trehalose biosynthesis, glycogen mobilisation, plant pathogenicity, sporulation, activation of the pentose phosphate pathway and nitrate utilisation gene expression are independent processes, each affected by Tps1.

An accumulating body of evidence in plants points to trehalose and T6P biosynthesis and metabolism being highly integrated with plant development, a relationship that was somewhat unexpected (Eastmond *et al*, 2002). Tps1 mutations in *Arabidopsis thaliana*, for example, lead to embryonic lethality due to loss in coordination in cell wall biosynthesis and cell division, indicating a key role for trehalose metabolism in plant embryonic development (Gómez *et al*, 2006). T6P levels have also been shown to affect starch synthesis in *Arabidopsis* by post-translational redox activation of ADP-glucose pyrophosphorylase (Kolbe *et al*, 2005), while in maize, a recent study demonstrated that T6P phosphatase encoded by *TPS2* is necessary for inflorescence architecture (Satoh-Nagasawa *et al*, 2006). Interestingly, transcriptional profile analysis of *Arabidopsis* in response to changes in nitrate availability, indicates an effect on the expression of trehalose metabolic genes (Scheible *et al*, 2004). Our identification of a role for Tps1 in *M. grisea* in regulating NADPH levels, the oxidative pentose phosphate pathway, and expression of nitrogen source utilisation and virulence genes show how trehalose biosynthesis might act in regulating complex developmental processes in multicellular eukaryotes such as plants and fungi.

Materials and methods

Fungal strains, manipulations and physiological tests

All *M. grisea* strains used in this study are derived from Guy-11. Standard procedures of *M. grisea* growth, maintenance, appressoria formation and transformation were performed as described previously (Crawford *et al*, 1986; Talbot *et al*, 1993). Gel electrophoresis, restriction enzyme digestions, ligations, DNA and RNA gel blot hybridizations were performed using standard procedures (Sambrook *et al*, 1989). DNA and RNA probes were radiolabelled using the random primer method (Feinberg and Vogelstein, 1983).

Strains were grown on complete medium (CM)-containing 1% (w/v) glucose, 0.2% (w/v) peptone, 0.1% (w/v) yeast extract and 0.1% (w/v) casamino acids—or GMM—containing 10% glucose and 0.6% sodium nitrate—unless otherwise stated, as described in Foster *et al* (2003).

Rice plant infections were made as described previously (Talbot *et al*, 1993). For additional information, see Supplementary data. Nucleic acid, trehalose and sugar phosphate extractions were performed as described in Supplementary data.

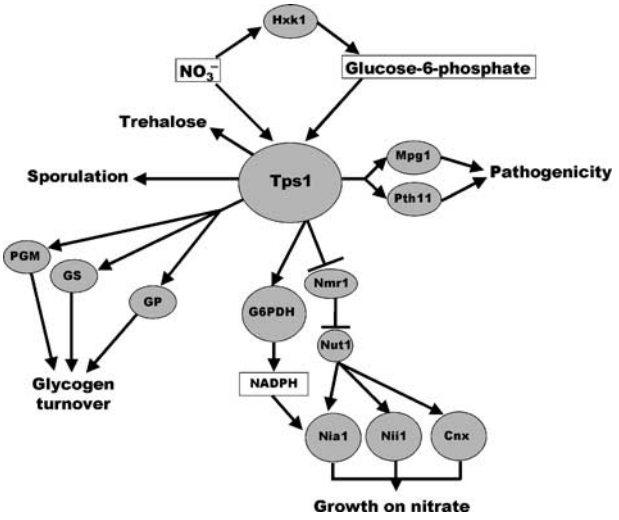


Figure 8 Model for the role of Tps1 in *Magnaporthe grisea*. In response to G6P and nitrate, Tps1 coordinates nitrate utilisation by a genetic response involving *NMR1* and a post-translational effect on NADPH levels via stimulation of G6PPDH activity. Tps1 also regulates glycogen metabolism based on its effect on G6P levels and independently controls sporulation-related functions and virulence-associated gene expression. Protein names are explained in the text, except the following: PGM, phosphoglucomutase; GS, glycogen synthase; G6PDH, G6P dehydrogenase; GP, glycogen phosphorylase; Nia1, nitrate reductase and Nii1, nitrite reductase.

Enzyme activity assays

All enzymatic assays were performed at 22°C. All assay components were purchased from Sigma, except NADH, NADPH and NADP (Calbiochem). Enzyme activities were determined spectrophotometrically in triplicate, and are expressed as the concentration of product formed in 1 min by total cell protein from 1 mg of mycelium. For full experimental protocols, see Supplementary data.

Metabolite identification and quantification

Trehalose was detected by hydrolysis to glucose using 3 mU of porcine kidney acidic trehalase (Sigma), followed by determination of glucose concentration using a diagnostic kit (Roche). The concentration of glucose from the trehalase-treated extract was compared to glucose from non-treated control, the difference representing the concentration of glucose liberated from trehalose.

Sugar phosphates were quantified using HPAE-PAD analysis on mycelial extracts using a BioLC system (Dionex). See Supplementary data for details. Mycelial glycogen concentration was determined enzymatically from mycelium, as described in Supplementary data. Glycogen was visualised in appressoria using an iodine stain consisting of 60 mg KI and 10 mg I_2, per millilitre, in distilled water. Samples were observed with a Nikon Optiphot microscope connected to a Nikon FX-35 camera. Mycelial concen-

trations of NADPH and ATP were determined enzymatically, as described in Supplementary data.

Protein modelling

Protein modelling of *M. grisea* Tps1 and *E. coli* otsA (Gibson *et al*, 2002) was performed as described in Supplementary data.

Construction of targeted gene replacement vectors and gene expression studies

A full description of all plasmids used in this study, and their construction, is provided in Supplementary data. *MPG1* gene expression was examined by Northern blot analysis. *NMR1, PTH11, NIA1, NII1* and β-tubulin gene expression was examined using RT–PCR (Clontech). Following PCR, quantification of the resulting amplicons used the ImageMaster TotalLab software programme and normalisation of expression data was achieved by quantification of actin gene expression. For full details, see Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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