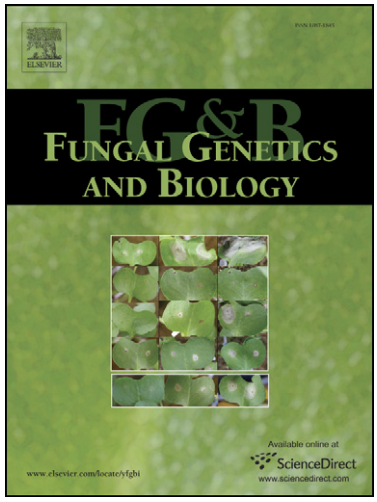

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Trehalose biosynthesis is involved in sporulation of *Stagonospora nodorum*

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Abstract

Stagonospora nodorum is a necrotrophic fungal pathogen that is the causal agent of leaf and glume blotch on wheat. *S. nodorum* is a polycyclic pathogen, whereby rain-splashed pycnidiospores attach to and colonise wheat tissue and subsequently sporulate again within 2-3 weeks. As several cycles of infection are needed for a damaging infection, asexual sporulation is a critical phase of its infection cycle. A non-targeted metabolomics screen for sporulation-associated metabolites identified that trehalose accumulated significantly in concert with asexual sporulation both *in vitro* and *in planta*. A reverse-genetics approach was used to investigate the role of trehalose in asexual sporulation. Trehalose biosynthesis was disrupted by deletion of the gene *Tps1,* encoding a trehalose 6-phosphate synthase, resulting in almost total loss of trehalose during *in vitro* growth and *in planta*. In addition, lesion development and pycnidia formation were also significantly reduced in *tps1* mutants. Reintroduction of the *Tps1* gene restored trehalose biosynthesis, pathogenicity and sporulation to wild-type levels. Microscopic examination of *tps1* infected wheat leaves showed that pycnidial formation often halted at an early stage of development. Further examination of the *tps1* phenotype revealed that *tps1* pycnidiospores exhibited a reduced germination rate while under heat stress, and *tps1* mutants had a reduced growth rate while under oxidative stress. This study confirms a link between trehalose biosynthesis and pathogen fitness in *Stagonospora nodorum.*

1 Introduction

Stagonospora nodorum is a necrotrophic fungal pathogen that is the causative agent of Stagonospora nodorum blotch of wheat. This disease costs the Australian wheat industry up to AUD $60 million per year in losses, and also has a significant effect on wheat production in North America (Bhathal et al., 2003; Brennan and Murray, 1998). The disease is typically initiated by wind-borne sexual ascospores that land on the leaf surface, but can also be initiated from infected seed (Solomon et al., 2006a). Under ideal conditions, multiple rounds of asexual reproduction will occur on the plant host during one growing season. This allows the pathogen to keep pace with the growth of the host, promoting colonisation of the flag leaf and glume. The polycyclic nature of *S. nodorum* infection means that successful asexual reproduction is a key factor in a damaging infection. With the goal of controlling the pathogen in mind, we have used a broad range of molecular techniques to elucidate the mechanisms of *S. nodorum* pathogenicity. This has revealed many factors with an influence on sporulation. A cohort of signalling proteins, the G-alpha subunit Gna1, the mitogen-activated protein kinase Mak2, and the calcium/calmodulin-dependant kinases CpkA and CpkC have been shown to be involved in asexual sporulation in *S. nodorum* (Solomon et al., 2006c; Solomon et al., 2004b; Solomon et al., 2005b). Work studying the catabolism/anabolism of mannitol confirmed a requirement for mannitol 1-phosphate dehydrogenase, and mannitol dehydrogenase activities for asexual sporulation (Solomon et al., 2005a; Solomon et al., 2006d). Interestingly, trehalose was more abundant in strains lacking mannitol 1-phosphate dehydrogenase. Recently, the short-chain dehydrogenase Sch1, which is up-regulated by Gna1-dependant signalling, has been shown to be required for asexual sporulation (Tan et al., 2008). Also of interest is the mode of action of malayamycin, a novel antifungal molecule

which, in *S. nodorum*, acts primarily though inhibition of sporulation (Li et al., 2008; Loiseleur et al., 2007).

A non-targeted metabolomics study was completed to further characterise metabolic functions required for sporulation. The disaccharide trehalose was found to markedly increase during sporulation both *in vitro* and *in planta*. *S. nodorum* strains lacking a predicted trehalose 6-phosphate synthase, *Tps1*, were shown to be affected in their ability to develop lesions and were unable to develop pycnidia and pycnidiospores at the wild-type rate. Trehalose levels *in planta* and *in vitro* were greatly reduced in *tps1* strains, and the ability to resist oxidative stress was also diminished.

2 Materials and methods.

2.1 Strains and culture conditions.

S. nodorum SN15 was obtained and cultured on CZV8CS medium or minimal medium as described previously (Solomon et al., 2004a).

2.2 Infection conditions.

Whole plant spray and detached leaf assays were conducted as described previously (Solomon et al., 2004a; Solomon et al., 2004b). Infections were conducted at least twice.

2.3 Gene deletion and complementation constructs.

The pTPSKO knockout plasmid was constructed as follows: A 1056 bp region 5' of the *Tps1* start codon was amplified by PCR with SN15 gDNA and primers TPSKO5'F (5'-CTCGAGAGATCTAATAGATGCCATAA-3') and TPSKO5'R (5'-

AAGCTTTGTCATGTTTGCGGTATATA-3') and an 1119 bp region 3' of the *Tps1* stop codon was amplified using primers TPSKO3'F (5'-GCAGAGCAGCTCCCTTGCCGTTCT-3') and TPSKO3'R (5'-GCGGCCGCTCTATAGATGGTGTACAGTC-3'). The flanks were separately cloned in pGEM-T-easy, creating the pGEM-TPS-5'and pGEM-TPS-3' plasmids. Both flanks were then subcloned into pBSK-phleo, with the 3' flank ligated into *Pst*I and *Not*I sites, and the 5' flank ligated into the *Xho*I and *Hin*dIII sites, respectively, creating the knockout construct pTPSKO.

For the creation of the *Tps1* complementation construct, the *Tps1* locus was amplified with primers TPScompF (5'-CAGAAACGCTGGTGAAAGTAAAAGATTTGGTGGTGTTGTTTCCT -3') and TPScompR (5'- GGTATCTGAATGCTGCATTTT -3'). A region conferring hygromycin resistance was amplified from the plasmid pAN7-1 with primers pAN7F (5'- TGTAGAAAAATGTGACGAACTCGTG -3') and pAN7R (5'-TCTTTTACTTTCACCAGCGTTTCTG -3') and fused to the *Tps1* amplicon during an overlap PCR. The underlined sequence in TPScompF anneals to the 3' end of the amplified hygromycin resistance cassette during overlap PCR.

2.4 Transformation of S. nodorum.

S. nodorum protoplasts were made as previously described (Solomon et al., 2004b). pTPSKO was linearised by digestion with *Xho*I endonuclease and 5 µg used per transformation. The tps1comp PCR construct was gel extracted and eluted in STC buffer prior to transformation. *S. nodorum* protoplasts were transformed by PEG-mediated transformation as previously described (Solomon et al., 2006c). Genomic

DNA from transformants was screened by PCR amplification of the *Tps1* locus. In the case of the *tps1* knockout a small number of strains were further analysed by Southern blot.

2.5 Southern Blot analysis.

Genomic DNA (5 µg) was digested with *Nco*I and separated by gel electrophoresis before transfer to positively charged nylon membrane, as previously described (Solomon et al., 2004a). A 150 bp region of the pTPSKO 3' flank was amplified by PCR using primers TPSKO3'F and TPS-Probe-3' and randomly labelled with DIG High Prime (Roche) for use as a hybridisation probe, as per manufacturers instructions. The probe was predicted to hybridise to a 0.9 kb fragment in *Tps1* strains and to a 1.5 Kb fragment in *tps1::Phleo* strains; and to both a 0.9 kb and a 1.5 Kb fragment in strains containing an ectopic integration of pTPSKO.

2.6 Sporulation assay

Sporulation assays *in vitro* were conducted by culturing strains on solid media spot-inoculated with 5×10^5 spores and incubated at 20 ºC for 3 weeks before pycnidiospores were harvested and counted. Sporulation *in planta* was conducted with a latent period assay, as follows; Two-week-old wheat plants were sprayed with spores from each strain and infection was allowed to progress for 7 days before infected leaves were excised and mounted in benzimidazole agar. Infections were incubated at 20 ºC for 7 days to encourage sporulation before spores were isolated from the leaves (n = 3). Statistical groupings were determined using JMP software and the Tukey-Kramer test (P=0.05). Assays were conducted at least twice with comparable results achieved across experiments.

2.7 Liquid culture growth assay.

Minimal medium (200 µL) was inoculated with 5000 pycnidiospores as described previously (Solomon et al., 2004a). Eight replicates were performed per strain, in a 96-well microtitre plate format with incubation at 20 ºC for 7 days. The net change in OD 595 nm for each culture was recorded and used to infer growth of the strain. For oxidative stress assays, minimal medium was supplemented with t-butyl peroxide, and growth was expressed as a proportion of that achieved in the absence of oxidant. This experiment was repeated twice with similar results achieved.

2.8 Germination assay.

Approximately 5000 pycnidiospores were deposited on glass slides coated in 1 % (w/v) agarose and incubated in a humidified chamber. Germ tube formation was observed using light microscopy. The percentage of pycnidiospores with a germ tube was calculated from a minimum of 100 spores, with three replicate slides prepared. This experiment was repeated twice with similar results achieved.

2.9 Gas chromatography-mass spectrometry (GC-MS).

Extraction of polar metabolites and GC-MS was performed as previously described by Solomon et al (2006). All samples were normalised to a ribitol internal standard. The resulting signal was then calculated relative to the weight of extracted tissue, except for *in vitro* analysis of *Tps1*-9-12 (Figure 3B) where the total chromatogram area was used to normalise trehalose to the total amount of extractable metabolites. Selective ion monitoring (SIM) GC-MS was performed with the following changes. Mass spectral ion acquisition was changed from scan mode to SIM mode. Three ions were monitored for quantification of three metabolites, m/z 319 (trehalose-TMS), m/z

217 (mannitol-TMS) and m/z 191 (ribitol-TMS). The dwell time for each ion was set to 20 ms.

2.10 Bioinformatic analysis.

The ClustalX algorithm was used to create multiple sequence alignments. The TreeView program was used to display the resulting phylogenetic trees.

3 Results.

3.1 Trehalose is a sporulation-associated metabolite in S. nodorum.

A non-targeted analysis of SN15 metabolism during sporulation both *in vitro* and *in planta* was performed in order to identify sporulation-specific metabolites. For *in vitro* sporulation, SN15 was grown on solid minimal medium and tissue harvested prior to sporulation occurred (4 dpi), during the initiation of sporulation (11 dpi), and at the peak of asexual sporulation (18 dpi). For *in planta* sporulation, wheat cv. Amery was infected with SN15 in the latent period assay format, and lesions excised at 8 dpi, 10 dpi, and 12 dpi, representing pre-sporulation, mid-sporulation and peak-sporulation stages. Polar metabolites were extracted from tissues and analysed by GC-MS. Trehalose was identified as being specifically associated with asexual sporulation of SN15, both *in vitro* and *in planta* (Figure 1 A, B). *In vitro*, trehalose levels continually increased as the colony matured and produced pycnidia. *In planta*, a similar trend was observed, with no trehalose detected at 8 dpi when lesions were devoid of pycnidia, and successively greater amounts of trehalose measured as pycnidia developed. As the relationship between trehalose biosynthesis and sporulation of *S. nodorum* had not been studied previously, the hypothesis that

trehalose was produced during asexual sporulation was tested by a reverse-genetics approach.

3.2 Trehalose biosynthesis in S. nodorum.

The first committed step in trehalose biosynthesis in fungi is the formation of trehalose 6-phosphate from UDP-glucose and glucose 6-phosphate by trehalose 6-phosphate synthase (TPS). Trehalose 6-phosphate is subsequently dephosphorylated by trehalose 6-phosphate dephosphorylase (TPP). The reaction performed by TPS is a key metabolic control point (Thevelein and Hohmann, 1995), and was therefore selected for mutagenesis. The *S. nodorum* SN15 genome sequence was searched for genes predicted to encode such an enzyme. A TBlastN blast search of the *S. nodorum* genome sequence (Hane et al., 2007)) with the *S. cerevisiae* Tps1 protein sequence (Genbank accession AAT27376) identified three regions of similarity, corresponding to the predicted genes SNOG_09603, SNOG_03369 and SNOG_02747. A BlastP alignment of the *S. nodorum* sequences to the *S. cerevisiae* Tps1 revealed SNOG_09603 was a reciprocal best hit with ScTps1 with 63% identity, while SNOG_03369 and SNOG_02747 were less well aligned with 37% and 38 % identity, respectively. BlastP comparison of the SNOG_09603 predicted protein sequence with the Swissprot database revealed many strong alignments with other experimentally validated fungal alpha-alpha-trehalose 6-phosphate synthases. The top hit to SNOG_09603 was alpha,alpha-trehalose-phosphate synthase (Genbank accession O59921) from *Aspergillus nidulans,* sharing 72% identity with SNOG_09603 at the amino acid level.

A multiple sequence alignment was performed and visualised by an average-distance phylogenetic tree to define the sequence relationships between the three potential *Tps1* genes (Figure 2). The alignment included related fungal sequences with experimental evidence for the functions of interest. The alignment grouped SNOG_09603 with verified Tps1 proteins. The other two potential Tps1 sequences, SNOG_02747 and SNOG_03369, grouped apart with the trehalose 6-phosphate phosphorylases and Tps3 regulatory proteins, respectively.

3.3 Disruption of the gene encoding trehalose 6-phosphate synthase.

To determine whether trehalose biosynthesis is required for sporulation of *S. nodorum*, the *Tps1* gene was deleted from *S. nodorum* SN15 using targeted gene replacement. The knockout vector, pTPSKO (Supplementary data), was linearised and used to transform SN15 protoplasts. A selection of phleomycin-resistant transformants were analysed by Southern blot to confirm the integration of the knockout construct at the *Tps1* locus (Supplementary data). Strains *tps1*-9, *tps1*-13, *tps1*-15 and *tps1*-21 were each shown to contain a single integration of pTPSKO at the *Tps1* locus. The knockout strains *tps1*-9, and *tps1*-13 were selected for phenotypic analysis. Two strains containing a single random integration of pTPSKO (ectopic integration) were identified, however, they were found to be unsuitable as controls during phenotypic analysis due to extremely variable behaviour. To confirm that the phenotypic changes observed in *tps1* strains were due to a single genetic lesion at the *Tps1* locus the wild-type gene was re-introduced back into *tps1*-9. The entire *Tps1* locus was amplified from SN15 genomic DNA with primers TPScompF and TPScompR. The 3.4 Kb region contained the Tps1 coding region along with 1.4 kb of promoter and 0.2 kb of terminator sequence. The Tps1 locus was fused to the

hygromycin resistance cassette by overlap PCR and the resulting amplicon was used to transform *tps1*-9 protoplasts to hygromycin resistance. Two complemented strains were analysed, *Tps1*-9-9 and *Tps1*-9-12

3.4 Tps1 is required for trehalose anabolism in vitro.

The ability of the *tps1* strains to synthesise trehalose was analysed during growth in culture. Trehalose was detected at low levels in SN15 cultures by 5 dpi, and once sporulation had occurred (at 20 dpi), a large amount of trehalose was produced (Figure 3A). Trehalose was not detected in the *tps1*-9 or *tps1*-13 strain at either timepoint. The trehalose content in *Tps1*-9-12 was determined after 5 days and 20 days growth on MM sucrose (Figure 3B). These assays showed that trehalose levels in the *Tps1*-9-12 complemented strain increased markedly by 20 dpi and were not significantly different to those found in SN15 cultures. In previously reported fungal *tps1* strains, concentrations of the glycolytic intermediate glucose 6-phosphate (G6P) have been shown to be altered by the loss of *Tps1* (Fillinger et al., 2001; Foster et al., 2003). At 5 dpi, the G6P levels in *tps1-9* and *tps1-13* were significantly higher than that observed in SN15 (Figure 3C). By 20 dpi G6P was reduced to trace amounts in all cultures. Trehalose levels were measured during *in planta* growth to determine whether the plant host affected the steady-state levels of trehalose in the pathogen. A detached leaf assay was performed and trehalose content of the lesion was analysed at 7 dpi (Figure 3D). The amounts of trehalose was markedly reduced in the mutants: *tps1*-9 was 0.6 %; *tps1*-13 was 0.9 % and the mock, 0.9 % of the wild type SN15 infection; In all analyses, mutants lacking the *Tps1* gene showed an almost total loss of trehalose, both in *vitro* and *in planta* growth situations.

3.5 Nutrient utilisation by tps1 strains.

As *tps1* strains in *M. grisea* have been reported to have significantly reduced growth rates on certain culture media (Foster et al., 2003), the ability of the mutants to utilise various carbon and nitrogen sources was determined. Defined liquid media were inoculated with pycnidiospores and incubated at 20 ºC for 7 days. Growth was not considered impaired by loss of the *Tps1* gene (Supplementary data).

3.6 The Tps1 gene is required for full pathogenicity and asexual sporulation in planta *and* in vitro.

The ability of the *tps1* mutants to cause disease on *Triticum aestivum* cv. Amery was tested by a whole-plant spray assay and a detached leaf assay. Firstly, two-week-old plants were sprayed with pycnidiospores from the *tps1* strains, SN15, or a mock inoculum. The visible disease symptoms were not significantly reduced in *tps1*-9 or *tps1*-13 (Supplementary Data). The rate of lesion expansion was then studied using the detached leaf assay. Lesion expansion was reduced after loss of the *Tps1* gene, as the two mutants produced lesions approximately 75 % of the size produced by wild-type after 7 days (Figure 4A), while in the complemented strains *Tps1*-9-9 and *Tps1*-9-12 lesion formation was found to be equivalent to that of SN15 (Figure 4B). In addition, the *tps1* strains were severely affected in their ability to produce asexual sporulation structures (Figure 4C and D). SN15 produced an average of 61 mature pycnidia per leaf, while *tps1*-9 and *tps1*-13 produced an average of 20 and 19, respectively. The pycnidia counts from the two complemented strains were not significantly different from those produced by SN15 (Figure 4E).

The ability of the *tps1* strains to sporulate *in vitro* was examined in the presence and absence of trehalose. When grown on MM sucrose, SN15 produced at least 20-fold more spores than the *tps1* strains (Table 1). Addition of trehalose increased the number of spores produced by SN15 but did not complement the mutant strains. When grown on complete media (CZV8CS) all strains recovered to produce greater amounts of spores than in the other two media, but the *tps1* strains showed the greatest increases. To characterise sporulation rates *in planta*, pycnidiospores were isolated from infected wheat leaves that had been allowed to complete the asexual cycle (Table 1). The assay showed that SN15 produced significantly more spores per leaf than either of the *tps1* mutants *in planta* but the difference between strains was reduced compared to sporulation *in vitro*.

3.7 Tps1 is required for development of pycnidia.

The *tps1* sporulation defect *in planta* was further studied by staining 7-day old infected detached leaves with Trypan blue, before observation by light microscopy (Figure 5). SN15-infected leaves contained many pycnidia, which stained as large, opaque, spherical structures (Figure 5A). In addition to densely stained pycnidia, widespread staining of hyphae within the leaf was visible in all SN15 examples. The *tps1*-9 and *tps1*-13 infections examined revealed an increased presence of immature pycnidia relative to SN15. The *tps1* infections occasionally produced pycnidial primordia with distinct margins, and commonly contained less-developed early-stage pycnidial development structures such as hyphal knots. The immature pycnidia identified in *tps1*-9 and *tps1*-13 were not pigmented, while most pycnidia in the SN15 sample were fully developed and melanised.

Considering the observed reduction in sporulation in concert with a reduced level of trehalose in whole cultures of *tps1* mutants, it was hypothesised that trehalose was accumulating within the wild-type pycnidiospore. (Figure 5). Cultures were grown on a complete medium (CZV8CS) or minimal medium (MM), before pycnidiospores were separated from the main fungal biomass and analysed using SIM-mode GC-MS. Due to the difficulty in analysing small quantities of spores, sample-to-sample variation was such that precise measures were not reliable, therefore the data has been regarded as qualitative rather than quantitative (Table 2). When grown on complete media, SN15 clearly contained much larger amounts of trehalose than either of the mutants, while on minimal medium the difference was reduced. The difference between SN15 and the *tps1* strains was not consistent, but it was clear that there were significant amounts of trehalose in the pycnidiospore and that SN15 was able to produce it in much greater quantities. Growth on minimal medium resulted in a general reduction in trehalose content in all strains, but the *tps1* strains had consistently lower spore trehalose levels in comparison to SN15.

3.8 Stress response in the tps1 strains.

Tps1 mutants have been shown to have reduced pathogenicity and sporulation compared to the wild-type strain and to contain less trehalose. A possible explanation for the reduction in pathogenicity and sporulation was that the *tps1* strains were unable to cope with stresses encountered during infection. As *Tps1* in *Botrytis cinerea* has been implicated in the heat stress response (Doehlemann et al., 2006), pycnidiospores were germinated on agar slides at a range of temperatures in order to gauge their ability to function while under heat stress (Figure 6). Significant differences in germination rates were observed between strains at 35 ºC, where SN15

(29 %) had a significantly higher germination ratio than either *tps1*-9 (12 %) or *tps1*-13 (7 %). The other temperatures did not differentiate between the strains.

The ability of the *tps1* mutants to cope with oxidative stress, was examined using t-butyl-peroxide as an oxidant. Optical density of the cultures was used to infer growth rates and it was found that *tps1* cultures grown in 0.03 mM or 0.1 mM t-butyl-peroxide grew more slowly than wild-type cultures (Figure 7). When trehalose was added to the medium, 0.1 mM t-butyl-peroxide no longer differentiated the *tps1* strains from the wild-type (Figure 7B).

4 Discussion

4.1 Trehalose in fungi

Trehalose is a characteristic metabolite of fungi and is much more abundant and frequent in this kingdom than elsewhere. However the role(s) of trehalose remain ill defined. It is a key metabolite in the sporulation of several fungi where it can accumulate to high concentrations in hyphae and spores. It also accumulates in response to environmental stress. In *Saccharomyces cerevisiae* trehalose accumulates to high levels in stationary-phase cultures and spores, and in cells subjected to heat shock (Singer and Lindquist, 1998). Blocking trehalose biosynthesis resulted in increased susceptibility to heat and oxidative stresses. In filamentous fungi, many studies have shown that trehalose is important for a variety of functions associated with survival and pathogenicity. In *Aspergillus nidulans*, loss of trehalose biosynthesis resulted in a reduction in the ability to cope with mild heat and oxidative stress (Fillinger et al., 2001). Studies have also been performed in the plant pathogens *Magnaporthe grisea* and *Botrytis cinerea*. In *M. grisea* Foster et al (2003) showed

the *Tps1* gene was required for trehalose biosynthesis and full pathogenicity. The loss of pathogenicity was attributed to the failure of the pathogen to accumulate trehalose in conidia and subsequent inability to form appressorium and penetrate the rice cuticle. In the same study, the neutral trehalase Nth1 was shown to be required for degradation of trehalose stored in conidia and for full invasive growth throughout the host tissues. In *Botrytis cinerea* removal of *Tps1* effected a reduction in trehalose biosynthesis resulting in reduced viability of conidia under normal growth conditions and at higher temperatures (Doehlemann et al., 2006).

4.2 *The Tps1 gene is required for trehalose biosynthesis, full pathogenicity, and asexual sporulation* in planta.

The *S. nodorum* genome was found to contain a homologue of the *S. cerevisiae Tps1* gene. The role of the putative *S. nodorum Tps1* gene was investigated by gene deletion and complementation. Quantitation of trehalose during growth *in vitro* confirmed that *tps1* strains were unable to synthesise trehalose. Restoration of the *Tps1* locus to a *tps1* strain resulted in restoration of trehalose to wild-type levels during growth in culture. The trace levels of trehalose that were found in isolated pycnidiospores of *tps1* strains was similar to previously reported *tps1* mutants. Deletion of *Tps1* in *M. grisea* did not completely remove trehalose from mycelial samples (Foster et al., 2003), while Doehlemann *et. al.* (2006) reported that in *Botrytis cinerea* traces of trehalose remained in conidia of a *tps1* strain. It is possible that other disaccharide synthesising enzymes have non-specific activities and may produce a small amount of trehalose, or trehalose-like sugars. The GC-MS technique used to quantify trehalose could potentially confuse low-abundance disaccharides if they had very similar retention times. There are two other trehalose biosynthetic

pathways described in the literature other than the OtsA pathway typical of fungi. The TreY-TreZ and the TreS pathways produce trehalose from glycogen and maltose, respectively, and can be found in bacteria and archaea (Elbein et al., 2003; Qu et al., 2004). Homologues of the protein-encoding genes for these three enzymes were not found in the *S. nodorum* genome. It is most probable that the trace levels of trehalose present in *tps1* spores was concentrated in sporulation structures after being scavenged from the environment.

In previously reported fungal *tps1* strains, the concentration of the glycolytic intermediate glucose 6-phosphate (G6P) was altered by the loss of *Tps1* in *A. nidulans*, *M. grisea*, and *S. cerevisiae* (Fillinger et al., 2001; Foster et al., 2003; Van Aelst et al., 1993). This glycolytic intermediate accumulated in yeast and in *A. nidulans tps1* strains (Fillinger et al., 2001). While yeast mutants suffered from depleted ATP levels and were unable to grow on glucose, *A. nidulans* mutants did not have reduced ATP levels and could utilise both glucose and fructose as a carbon source. In *S. nodorum* we have shown that the concentration of G6P was significantly raised in *tps1* strains, which could be due to a lack of Tps1 activity resulting in a surplus of G6P as occurred in other *tps1* mutants.

4.3 Tps1 is dispensable for lesion formation by S. nodorum

Tps1 mutants were shown to form lesions on wheat leaves to an extent comparable with wild-type. Lesion formation was determined by whole plant spray (WPS), which showed that the pathogenicity, or lesion formation was not statistically different to that of SN15 on entire seedlings. However, lesion expansion or development, as defined during a during a detached-leaf assay, was reduced to

approximately 75% of that achieved by SN15. The wild-type expansion rate was restored in the complemented strains *Tps1*-9-9 and *Tps1*-9-12. The reduction in lesion expansion in the mutants may be caused by an attenuated response to plant defences, or perhaps a decrease in the aggressiveness of the attack. It may be that trehalose biosynthesis helps *S. nodorum* colonise new tissue, but it is certainly not essential for penetration of the leaf, or colonisation of the leaf interior. This phenotype is most similar to that described for *B. cinerea tps1,* in which lesion development was not affected at all, but different to *M. grisea* where *Tps1* was required to generate appressoria turgor pressure and therefore cuticle penetration (Doehlemann et al., 2006; Foster et al., 2003). In *Cryptococcus neoformans*, a fungal pathogen of mammals, *Tps1* was shown to be required for virulence on rabbits and mice *(Petzold et al., 2006).* It appears that *Tps1* is required for many different roles across different organisms, even within fungal ascomycetes.

4.4 The tps1 strains exhibit a significant reduction in sporulation.

The most visible effect of deletion of *Tps1* was an attenuated maturation of pycnidia. Pycnidia development during *tps1*-9 and *tps1*-13 growth *in planta* appeared to arrest prior to differentiation of immature pycnidia into conidiogenous cells (Douaiher et al., 2004). The immature pycnidia were not visible in unstained samples, confirming that melanisation had not yet occurred. It may be that accumulation of trehalose is a developmental checkpoint for melanin formation in pycnidia (Tan et al., 2008). While the ability to produce pycnidia was not completely eliminated in the *tps1* strains, the few pycnidia that were produced appeared smaller and did not release mature spores onto the leaf surface. Pycnidiospore formation was more severely affected *in vitro* than that *in planta,* even when trehalose was added exogenously.

This observation is similar to the *tps1* strain of *M .grisea* where a pathogenicity defect was not restored by exogenous trehalose (Foster et al., 2003). The sporulation rates of wild-type and *tps1* strains were more similar on CZV8CS complete medium. This was expected, as *S. nodorum* sporulates very well when grown on CZV8CS. CZV8CS was analysed by GC-MS to see if trehalose was present in significant quantities, none was detected. It appears that CZV8CS can promote the sporulation of *tps1* strains, but in a manner independent of trehalose. The *in planta* sporulation assay showed that SN15 still produced significantly more pycnidiospores per lesion than either of the *tps1* mutants. However, the difference was reduced and *tps1* strains could produce spores at 60% of the wild-type rate, compared to approximately 10% *in vitro*. *In planta tps1* trehalose levels were very low, yet pycnidia and spore formation rates were higher than expected when compared to the situation *in vitro*. A simple explanation would be that sporulation *in planta* is induced by a more vigorous, and possibly more complex, set of signals than those triggering sporulation *in vitro*.

4.5 Strains lacking Tps1 are more sensitive to external stresses.

Defects in both sporulation and lesion development were observed in *tps1* mutants. These deficiencies may be due to an inadequate stress response. Oxidative and heat stress are both commonly associated with trehalose presence in fungi, for this reason both stresses were examined in the *tps1* background. *Tps1* strains exhibited increased sensitivity to heat stress during germination and growth in liquid culture. In addition, *Tps1* strains were shown to be more sensitive than SN15 to oxidative stress, and the effect could be attenuated by the addition of exogenous trehalose. In *S. cerevisiae* it has been shown that oxygen radicals damage amino groups on cellular proteins, and that trehalose reduces this damage (Benaroudj et al., 2001). It was suggested that

trehalose quenched the oxygen radicals in a similar manner to polyols such as mannitol. The exogenous addition of trehalose to the growth medium may have reduced the concentration of free radicals in solution without altering the trehalose concentration within the cell, however, it is still clear that the *Tps1* gene does provide a significant advantage in an oxidising environment. The theory that exogenous trehalose was unable to be transported into the cell is reinforced by the observed inability of exogenous trehalose to repair *tps1* sporulation defects, which would require trehalose import.

4.6 Nutrient utilisation by tps1 strains.

Loss of *Tps1* in *S. cerevisiae* caused mutants to become unable to grow on rapidly fermentable carbon sources such as glucose or fructose (Noubhani et al., 2000; Thevelein and Hohmann, 1995). In a different manner, the *M. grisea tps1* is unable to grow on nitrate with glucose as the sole carbon source (Foster et al., 2003) due to a defect in carbon/nitrogen regulation (Wilson et al., 2007). *S. nodorum Tps1* mutants were grown on minimal media with various simple carbon and nitrogen sources to determine if they were nitrate non-utilising (Supplementary data). No effects were observed. While this reported role for Tps1 was not thoroughly investigated in this study, our initial growth data indicates it does not have such a critical role in *S. nodorum*.

In summary, *Tps1* has been shown to be required for accumulation of trehalose to high levels during growth in culture and *in vitro*. *Tps1* was dispensable for lesion formation but its loss caused a significant reduction in sporulation both *in planta* and

in vitro. The reduction in sporulation may be due to the increased sensitivity of *tps1* strains to oxidative and heat stress. Trehalose has been shown to be a crucial pathogenicity and sporulation metabolite in *S. nodorum* and plays a role in its defence against stress.

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Figure Legends

Table 1

Sporulation of *tps1* strains *in vitro*.

Base media used were; MM sucrose (MM Suc), MM sucrose + trehalose (MM Suc + tre), CZV8CS (CZV8CS). Cultures were incubated at 20ºC for 3 weeks (n = 3). Pycnidiospore production by *tps1* strains *in planta*. Spore production by *tps1* strains was examined during a latent period assay (*In Planta*). Statistical significance was calculated using the Tukey-Kramer test (alpha 0.05), groups that share a common letter are not significantly different, as denoted under the heading Group.

Table 2

Trehalose content of the spores of *tps1* strains.

Spores were isolated from wild-type (SN15), and two deletion mutants (*tps1*-9, *tps1*-13), grown on CZV8CS or MM Sucrose media. Metabolites were extracted, separated and quantified by GC-MS in SIM mode. Samples were taken during heavy sporulation at 20 dpi. Approximate trehalose amounts are indicated as analysis of low-biomass samples was not absolutely consistent.

Figure 1

Trehalose is a sporulation-associated metabolite.

Trehalose abundance during sporulation was determined *in vitro* (A) and *in planta* (B). For *in vitro* sporulation, *S. nodorum* SN15 was grown on solid minimal medium for 18 days with tissue harvested at 4, 11 and 18 dpi. For *in planta* sporulation, wheat cv. Amery was infected with *S. nodorum* SN15 or a mock inoculum in a latent period

assay, lesions were harvested at 8, 10 and 12 dpi. Polar metabolites were extracted and analysed by GC-MS. Metabolite amounts are expressed as normalised peak areas/mg tissue. Means plotted +/- 1 SEM.

Figure 2

Phylogenetic tree of proteins involved in trehalose biosynthesis and breakdown in fungi.

Amino acid sequences were aligned with the ClustalX algorithm. Phylogenetic tree was built using TreeView software. Sequence labels contain the gene name, organism and genome gene identifier. The scale bar indicates the number of substitutions per site.

Figure 3

Key metabolite concentrations in *tps1* mutants during growth *in vitro* and *in planta*. *In vitro* samples were taken prior to sporulation (5 dpi) and during heavy sporulation (20 dpi) on minimal medium. Panels are as follows: Trehalose in *tps1* (A), Trehalose in *tps1* complemented strain (B), glucose 6-phosphate in *tps1* (C). Trehalose was also analysed *in planta* at 7 dpi during sporulation (D). Units are expressed as normalised area/mg dry tissue, except for panel B where units are normalised area as a percentage of the total peak area of the chromatogram. Means plotted +/- 1 SEM (n = 3).

Figure 4

Pathogenicity of *tps1* strains.

Wheat cv. Amery was infected with SN15, *tps1*-9 and *tps1*-13, or SN15, *Tps1*-9-9 and *Tps1*-9-12 in a detached leaf assay. Lesion size (A and B) and pycnidia formation (C, D, E) were recorded at 7 dpi. An asterisk indicates scores that were significantly different from SN15, P = 0.05, Means plotted +/- 1 SEM, (n = 5).

Figure 5

Microscopic morphology of *tps1* strains during sporulation *in planta*.

Leaves from 2-week-old wheat plants were excised, mounted in benzimidazole agar and spot inoculated with SN15 (A), mock solution (B), *tps1*-9 (C), tps1-13 (D, close-up *tps1*-9 (E) close-up *tps1*-13 (F). Infection was allowed to progress for 7 days before leaves were stained with trypan blue to reveal fungal structures under light microscopy. Leaves are positioned with the adaxial side in view. Arrowheads indicate fungal structures.

Figure 6

Germination rates of *tps1* strains under heat stress.

Spores were germinated on agarose slides for 6 hours at 20 ºC, 30 ºC, and 37 ºC. Germ tube formation by pycnidiospores was observed by light-microscopy. Means plotted +/- 1 SEM, (n = 3).

Figure 7

Growth rates of *tps1* strains under oxidative stress.

Cultures were grown in liquid culture for 7 days at 20 ºC in either MM Sucrose (A) or MM Sucrose+Trehalose (B) with 0 mM, 0.03 mM or 0.1 mM t-butyl-peroxide added. The increase in optical density at 595 nm was recorded at 7 dpi, and all values

normalised to growth in 0 mM t-butyl peroxide. An asterisk indicates instances where *tps1* strains were significantly different to wild-type. Means plotted +/- 1 SEM, (n = 6).

Table 1
Sporulation of *tps1* strains *in vitro*.

		Total spores			Relative to SN15	
Strain	Media	Average	Std Error	Group	Amount	Std Error
SN15	MM Suc	9.3E+07	8.6E+06	A	100%	9%
tps1-9	MM Suc	1.2E+06	8.1E+05	B	1%	1%
tps1-13	MM Suc	4.9E+06	2.4E+06	B	5%	3%
SN15	MM Suc + Tre	3.1E+07	7.6E+06	A	100%	25%
tps1-9	MM Suc + Tre	1.8E+04	8.8E+03	B	0.1%	0.03%
tps1-13	MM Suc + Tre	2.9E+06	1.3E+06	B	9%	4%
SN15	CZV8CS	1.7E+08	1.6E+08	A	100%	94%
tps1-9	CZV8CS	2.6E+07	4.9E+06	A	15%	3%
tps1-13	CZV8CS	2.5E+07	1.2E+07	A	15%	7%
SN15	*In Planta*	6.70E+04	1.70E+03	A	100%	3%
tps1-9	*In Planta*	4.50E+04	6.20E+03	B	67%	9%
tps1-13	*In Planta*	3.50E+04	7.00E+03	B	52%	10%
mock	*In Planta*	0	0	C	0%	0%

Table 2
Trehalose content of the spores of *tps1* strains.

	CZV8CS	MM
SN15	+++++	++
tps1-9	+	+
tps1-13	+	n.d

Figure1

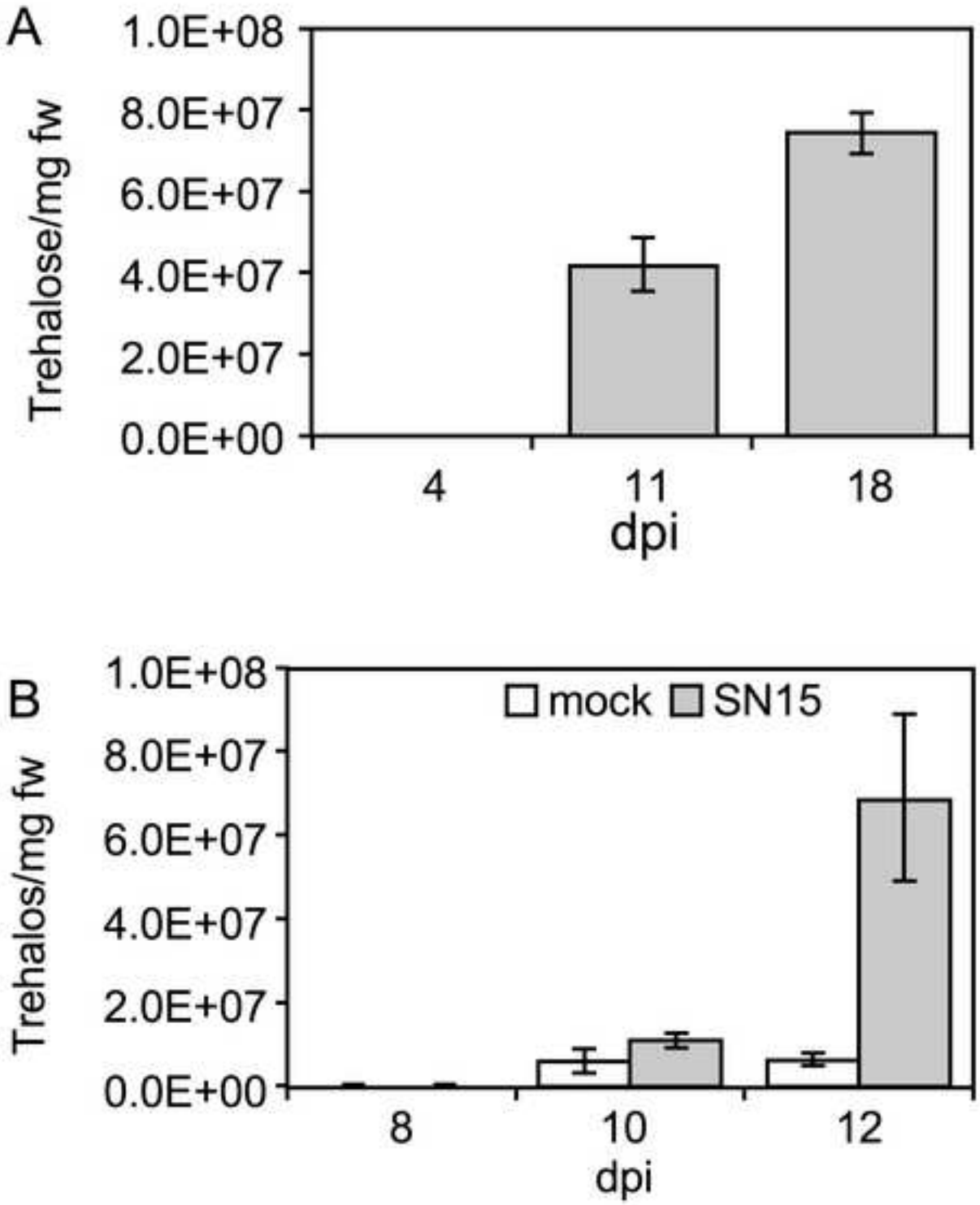

A
1.0E+08
8.0E+07
6.0E+07
4.0E+07
2.0E+07
0.0E+00
Trehalose/mg fw
4
11
18
dpi
B
1.0E+08
8.0E+07
6.0E+07
4.0E+07
2.0E+07
0.0E+00
Trehalos/mg fw
mock
SN15
8
10
12
dpi

Figure2

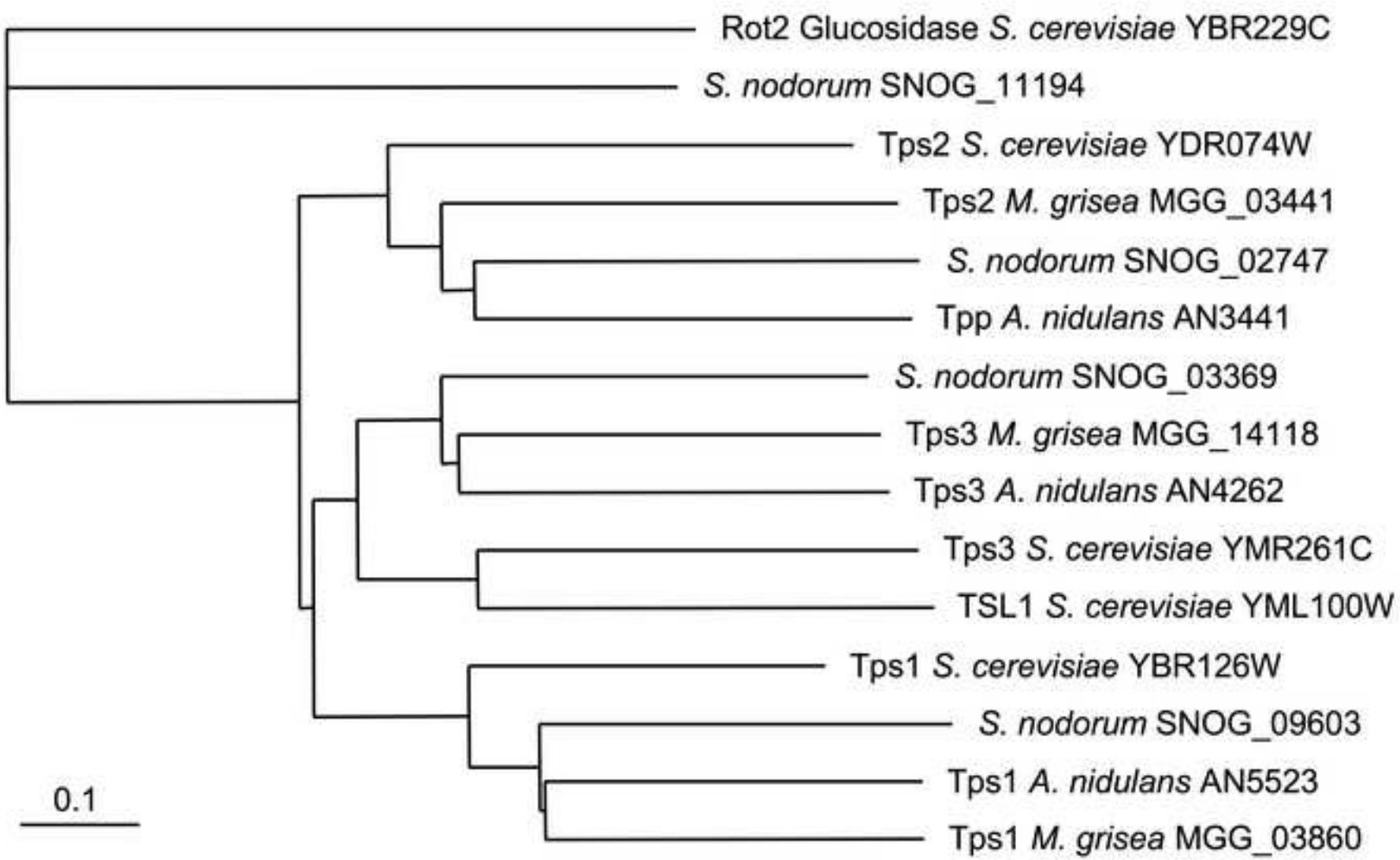

Rot2 Glucosidase S. cerevisiae YBR229C
S. nodorum SNOG_11194
Tps2 S. cerevisiae YDR074W
Tps2 M. grisea MGG_03441
S. nodorum SNOG_02747
Tpp A. nidulans AN3441
S. nodorum SNOG_03369
Tps3 M. grisea MGG_14118
Tps3 A. nidulans AN4262
Tps3 S. cerevisiae YMR261C
TSL1 S. cerevisiae YML100W
Tps1 S. cerevisiae YBR126W
S. nodorum SNOG_09603
Tps1 A. nidulans AN5523
Tps1 M. grisea MGG_03860
0.1

Figure 3

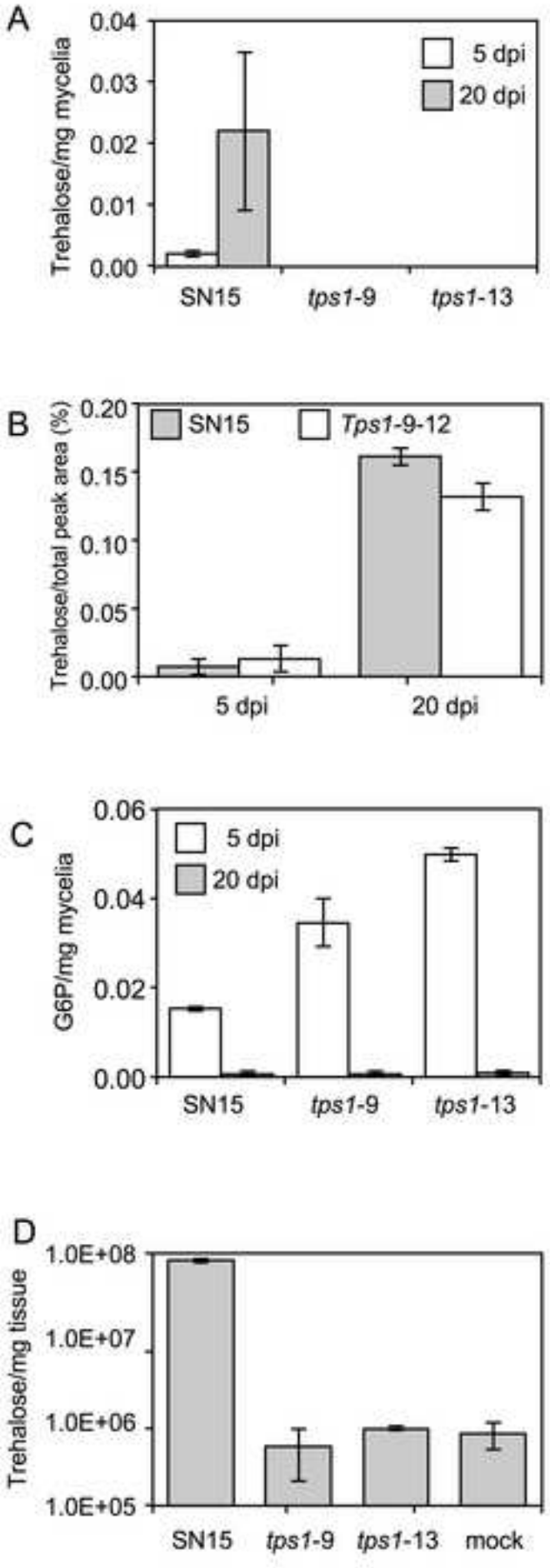

A
Trehalose/mg mycelia
0.04
0.03
0.02
0.01
0.00
5 dpi
20 dpi
SN15
tps1-9
tps1-13
B
Trehalose/total peak area (%)
0.20
0.15
0.10
0.05
0.00
SN15
Tps1-9-12
5 dpi
20 dpi
C
G6P/mg mycelia
0.06
0.04
0.02
0.00
5 dpi
20 dpi
SN15
tps1-9
tps1-13
D
Trehalose/mg tissue
1.0E+08
1.0E+07
1.0E+06
1.0E+05
SN15
tps1-9
tps1-13
mock

Figure 4

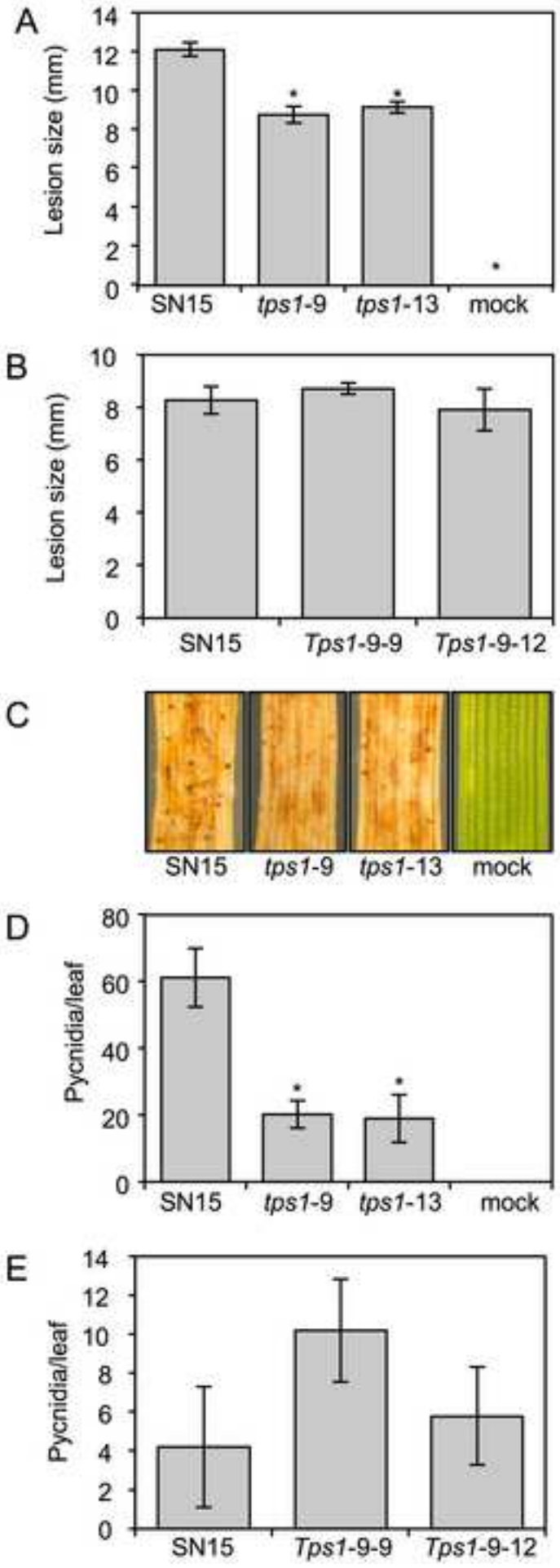

A
Lesion size (mm)
14
12
10
8
6
4
2
0
SN15
tps1-9
tps1-13
mock
B
Lesion size (mm)
10
8
6
4
2
0
SN15
Tps1-9-9
Tps1-9-12
C
SN15
tps1-9
tps1-13
mock
D
Pycnidia/leaf
80
60
40
20
0
SN15
tps1-9
tps1-13
mock
E
Pycnidia/leaf
14
12
10
8
6
4
2
0
SN15
Tps1-9-9
Tps1-9-12

Figure 5

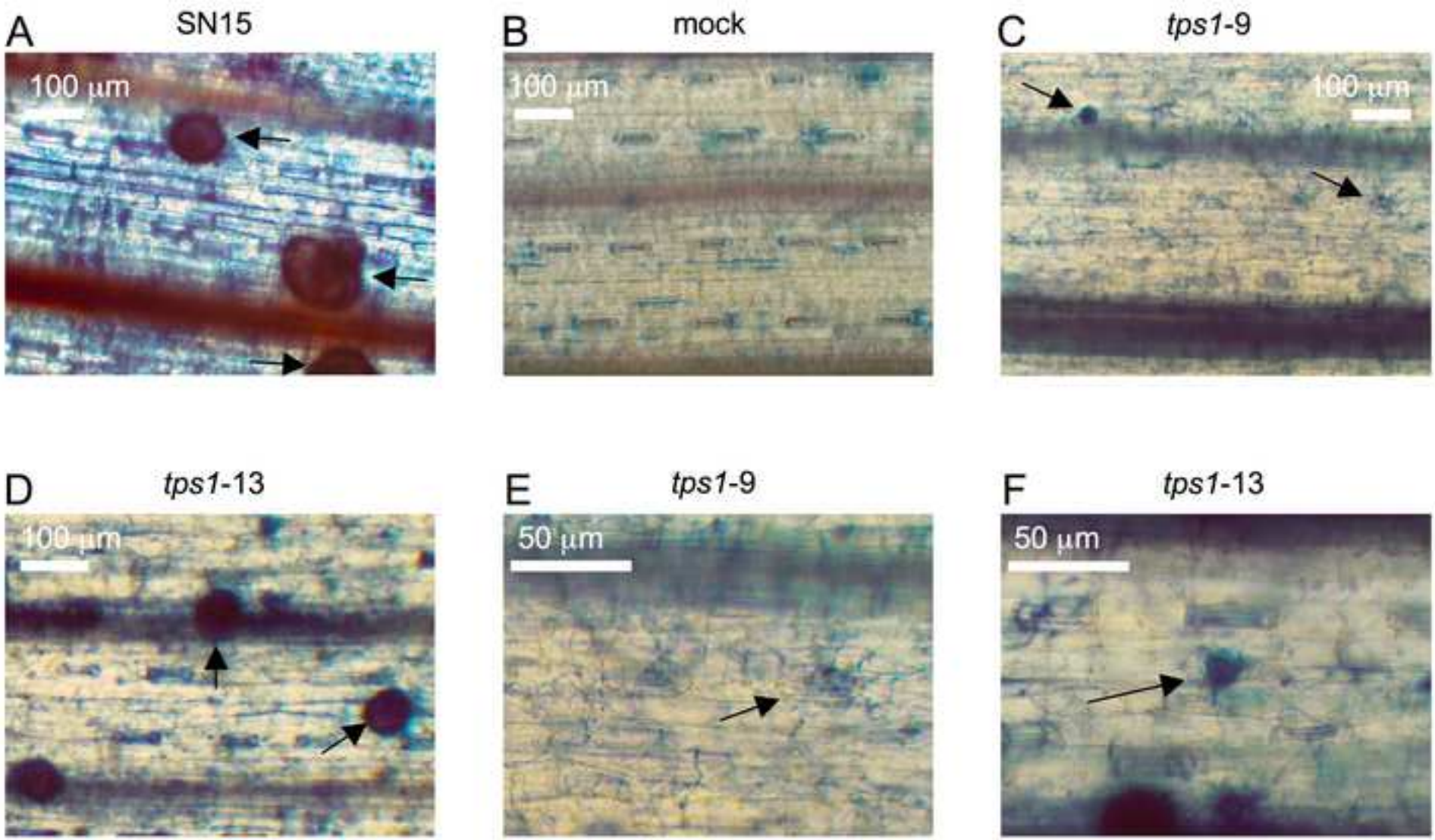

A
SN15
100 μm
B
mock
100 μm
C
tps1-9
100 μm
D
tps1-13
100 μm
E
tps1-9
50 μm
F
tps1-13
50 μm

Figure 6

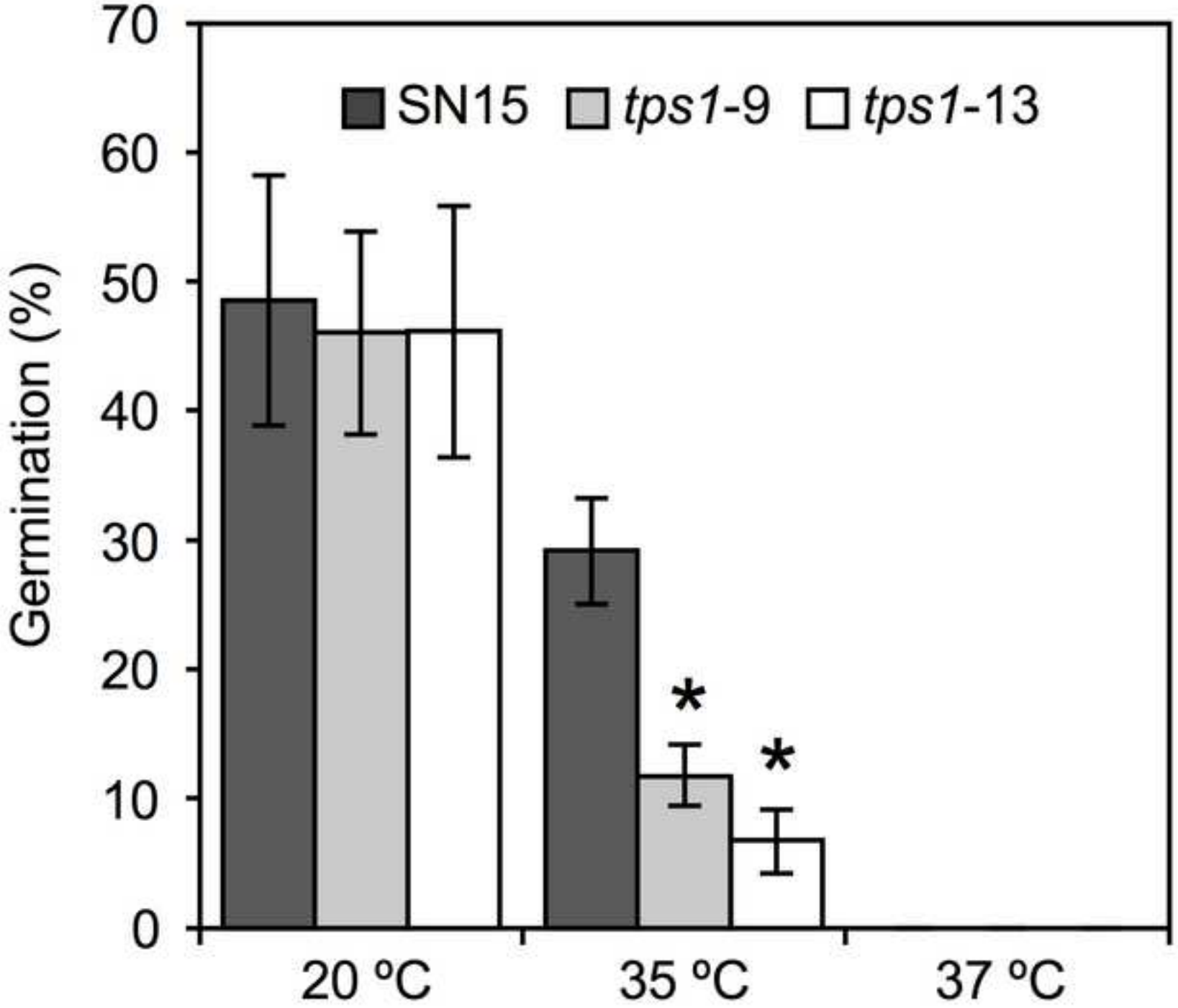
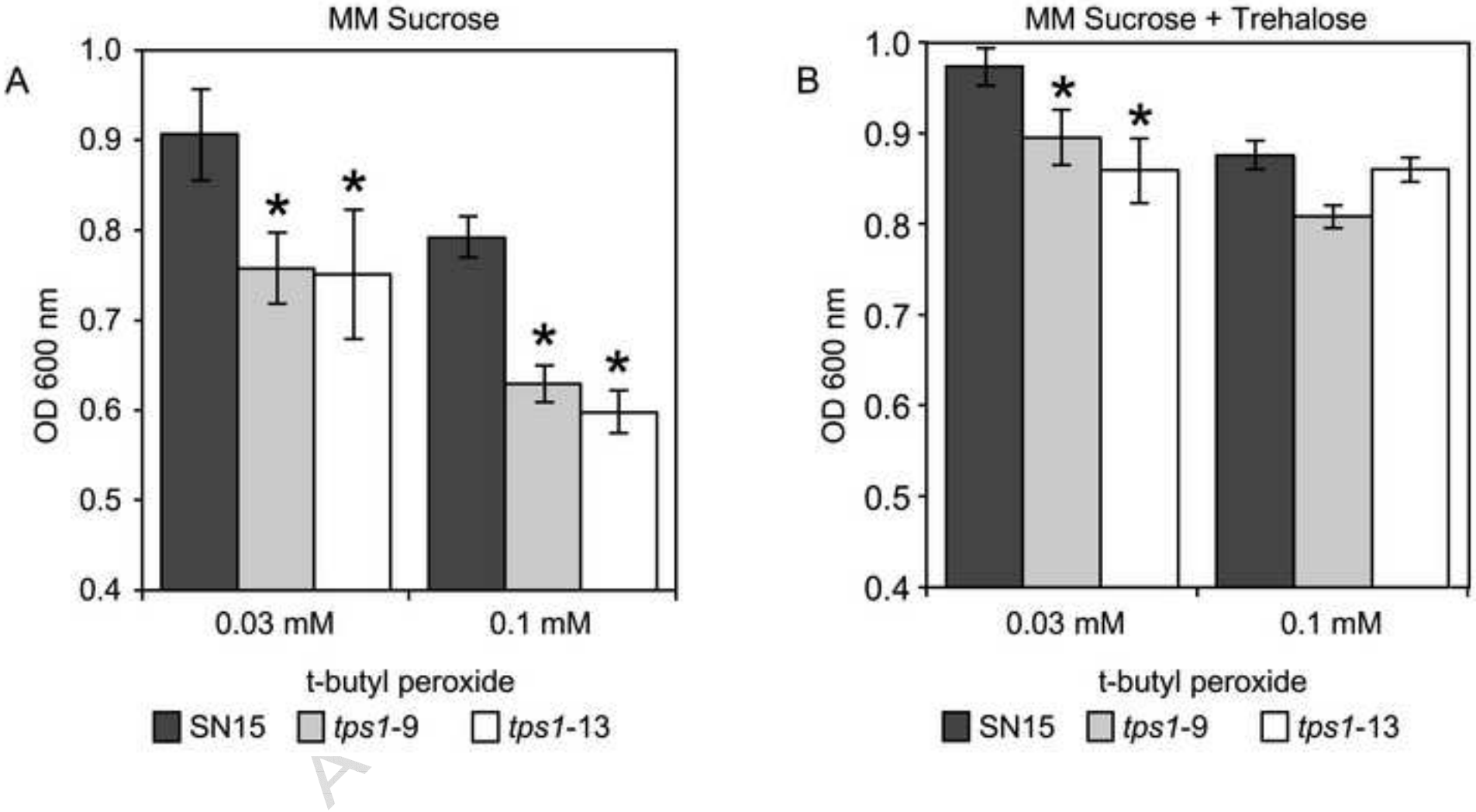

70
60
50
40
30
20
10
0
Germination (%)
SN15
tps1-9
tps1-13
*
*
20 ºC
35 ºC
37 ºC

Figure 7


A
MM Sucrose
OD 600 nm
1.0
0.9
0.8
0.7
0.6
0.5
0.4
*
*
*
*
0.03 mM
0.1 mM
t-butyl peroxide
SN15
tps1-9
tps1-13
B
MM Sucrose + Trehalose
OD 600 nm
1.0
0.9
0.8
0.7
0.6
0.5
0.4
*
*
0.03 mM
0.1 mM
t-butyl peroxide
SN15
tps1-9
tps1-13