Trehalose metabolism is important for heat stress tolerance and spore germination of *Botrytis cinerea*

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To analyse the role of trehalose as stress protectant and carbon storage compound in the grey mould fungus *Botrytis cinerea*, mutants defective in trehalose-6-phosphate synthase (TPS1) and neutral trehalase (TRE1) were constructed. The $\Delta tps1$ mutant was unable to synthesize trehalose, whereas the $\Delta tre1$ mutant showed elevated trehalose levels compared to the wild-type and was unable to mobilize trehalose during conidial germination. Both mutants showed normal vegetative growth and were not affected in plant pathogenicity. Growth of the $\Delta tps1$ mutant was more heat sensitive compared to the wild-type. Similarly, $\Delta tps1$ conidia showed a shorter survival under heat stress, and their viability at moderate temperatures was strongly reduced. In germinating wild-type conidia, rapid trehalose degradation occurred only when germination was induced in the presence of nutrients. In contrast, little trehalose breakdown was observed during germination on hydrophobic surfaces in water. Here, addition of cAMP to conidia induced trehalose mobilization and accelerated the germination process, probably by activation of TRE1. In accordance with these data, both mutants showed germination defects only in the presence of sugars but not on hydrophobic surfaces in the absence of nutrients. The data indicate that in *B. cinerea* trehalose serves as a stress protectant, and also as a significant but not essential carbon source for germination when external nutrients are low. In addition, evidence was obtained that trehalose 6-phosphate plays a role as a regulator of glycolysis during germination.

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INTRODUCTION

Trehalose [α -D-glucopyranosyl (1,1)- α -D-glucopyranoside], a non-reducing disaccharide, is absent in vertebrates, but occurs in nearly all other organisms including bacteria, yeasts, filamentous fungi and plants (Elbein *et al.*, 2003). In addition to its role as a carbohydrate storage compound, trehalose represents an important stress protectant and is involved in metabolic signalling and regulation of carbohydrate metabolism (Hounsa *et al.*, 1998; Arguelles *et al.*, 2000). The protective properties of trehalose during heat, dehydration and oxidative stresses are explained by its ability to stabilize the native conformation of proteins and membranes, but the molecular basis for this is not well understood (Singer & Lindquist, 1998). Contradictory models have been proposed to explain the stabilizing effects of trehalose, either by replacement of water molecules from the surface of biomolecules, or by entrapment of residual water molecules close to the biomolecule (Thevelein, 1996; Pereira *et al.*, 2004; Skibinsky *et al.*, 2005).

In yeast and *Aspergillus nidulans*, trehalose synthesis occurs in two steps, catalysed by trehalose-6-phosphate synthase (TPS), which condenses UDP-glucose and glucose 6-phosphate, and by trehalose-6-phosphate phosphatase (Gancedo & Flores, 2004; Fillinger *et al.*, 2001). Trehalose breakdown is catalysed by trehalases. Originally, two types of trehalases were described in fungi: so-called acid trehalases, with a low pH optimum, which are required for utilization of trehalose as a carbon source, and neutral trehalases, which mobilize intracellular trehalose (Thevelein, 1996; Amaral *et al.*, 1997). *Magnaporthe grisea*, which does not possess an acid trehalase, contains a neutral trehalase (NTH1), and a new type of fungal trehalase (TRE1) that has properties of both neutral and acid trehalases (Foster *et al.*, 2003). The phenotypes of mutants defective in trehalose metabolism have supported the role of trehalose as a protectant against heat and other stresses in yeasts and filamentous fungi (d'Enfert *et al.*, 1999; Fillinger *et al.*, 2001). In addition, trehalose has been shown to contribute to the energy requirements during conidial germination in *A. nidulans* (d'Enfert *et al.*, 1999). In the plant-pathogenic fungus *M. grisea*, trehalose synthesis and metabolism have been found to be required at different stages of plant infection (Foster *et al.*, 2003).

Abbreviations: CPT-cAMP, 8-(4-chlorophenylthio)-cyclic AMP; T6P, trehalose 6-phosphate; TPS, trehalose-6-phosphate synthase.

Botrytis cinerea, the causal agent of grey mould, infects more than 230 described host plant species (Jarvis, 1977). Its ubiquitous occurrence and its lifestyle as a facultative necrotrophic pathogen imply that it has to cope with a variety of biotic and abiotic stresses. In fact, its ability to produce and to withstand high concentrations of reactive oxygen species during pathogenesis has been well documented (Prins *et al.*, 2000; Rolke *et al.*, 2004). In addition, the grey mould fungus is equipped with a set of membrane transport proteins including several ABC and MFS transporters which confer resistance to a variety of toxic compounds (de Waard *et al.*, 2006; Hayashi *et al.*, 2002).

We have previously shown that germination of *B. cinerea* conidia occurs in response to either chemical or physical stimuli (Doehlemann *et al.*, 2005, 2006). Germination in response to the presence of carbon sources (e.g. glucose) was found to be dependent on a signalling pathway involving the $\alpha 3$ subunit of a heterotrimeric G protein (BCG3), cAMP and the mitogen-activated protein (MAP) kinase BMP1. In contrast, germination in response to hydrophobic surfaces – in the absence of nutrients – was not controlled by BCG3 and cAMP, but by another signalling pathway that was completely dependent on BMP1. Germination on full media, which does not require any of the above-mentioned signalling components, might be controlled by a third, so far poorly characterized signalling pathway (Doehlemann *et al.*, 2006).

In order to study the role of trehalose in *B. cinerea*, we have cloned and disrupted the genes encoding a neutral trehalase (*Bctre1*) and a trehalose-6-phosphate synthase (*Bctps1*). Characterization of the mutants and physiological studies revealed that trehalose is dispensable for pathogenesis, but important as a heat protectant. Furthermore, trehalose metabolism was shown to play a significant role during carbon source-induced germination, but not during

germination induced on hydrophobic surfaces in the absence of external nutrients.

METHODS

Fungal growth conditions and transformation. The *Botrytis cinerea* wild-type strain B05.10 was used in this work. The fungus was grown on tomato malt agar [1.5 % malt extract (Duchefa Biochemie) with 250 g homogenized tomato leaves l⁻¹, 1.5 % agar] and incubated for 5–14 days at 20 °C. Transformation of *B. cinerea* was performed as described previously (Reis *et al.*, 2005). For selective growth of transformants, HA agar (1 % malt extract, 0.4 % glucose, 0.4 % yeast extract, 1.5 % agar, pH 5.5) supplemented with hygromycin (Duchefa; 100 µg ml⁻¹) was used. After single-spore isolation, transformants were cultivated without selection on tomato malt agar.

Germination, plant infection and growth tests. Conidial germination assays, penetration assays on onion epidermis layers and plant infection tests were performed as described by Doehlemann *et al.* (2006). Growth tests providing osmotic or oxidative stresses were performed on plates containing minimal medium (Gamborgs B5 basal salt mixture; Duchefa), 0.5 % (w/v) glucose and 1.5 % agar. To provide oxidative stress, 2–5 mM H₂O₂ was added. Alternatively, the herbicide paraquat (Gramoxone; Syngenta) was added in concentrations of 5–10 mM. Osmotic stresses were provided by adding NaCl (0.5–1.5 M) or sorbitol (0.5–1.0 M). To test the growth of *B. cinerea* on different carbon sources in minimal medium, glucose was replaced by equal concentrations of the tested carbon source (fructose, sucrose, trehalose, sodium acetate, glycerol, yeast extract). To study the growth behaviour at different temperatures, *B. cinerea* was grown on HA agar.

Cloning and disruption of the *tps1* and *tre1* genes from *B. cinerea*. Starting out from EST sequences available for *B. cinerea* (*tps1*, AL110892; *tre1*, AL114821) and known sequences from the corresponding genes in *Aspergillus nidulans*, *Metarhizium anisopliae*, *Neurospora crassa* and *Magnaporthe grisea*, major regions of the *tps1* and *tre1* genes were amplified by PCR with combinations of gene-specific and degenerate primers (Table 1), and the products (*tps1*,

Table 1. Oligonucleotide primers

Primer (gene)	Sequence
TPS1-deg1 (<i>B. cinerea tps1</i>)	CCMATCACSATMAARCGNTC
TPS1-deg2	TTYTCCATGTCMTCNGGTGG
TPS1-KO1	CTCGTCGACATTGGTAACGGGCTTGAGTGG
TPS1-KO2	CTGGATCCTCATCGAATTCACCCGGTTCC
TPS1-KO3	CTGCGGCCGCTACACGCCCTTGAAGTCTTCC
TPS1-KO4	CTGCGGCCGCCGCAATGAAGGACACTCAAC
TPS1-8	CTGCGGCCGCTACACGCCCTTGAAGTCTTCC
TPS1-9	CTGCGGCCGCCGCAATGAAGGACACTCAAC
TRE1-deg1 (<i>B. cinerea tre1</i>)	TAYGGVTGGGAYAGYTAYATGG
TRE1-deg2	TTCCTYATHCARGTSGASCC
TRE1-KO1	GCACCCAGAATTCACAACCTTC
TRE1-KO2	TCGGATCCCCTCGAGAATATCTCTGGA
TRE1-KO3	TCGCGGCCGCGTGGTTGTCATCTGTGTCTTC
TRE1-KO4	CTGCGGCCGCGTGCAGTTGGATTAGACCGAC
HYG-1 (<i>E. coli hph</i>)	TCGCGGCCGCTGCAGCTGTGGAGCCGCATTC
HYG-2	CTGCGGCCGCCATGAATTGAAGCGGCACCTGGC

1567 bp; *tre1*, 2628 bp) were cloned into the vector pBS(+). For targeted disruption of the genes, a hygromycin resistance cassette was introduced by an inverse PCR strategy (see Fig. 2). For *tps1* disruption, inverse PCR was performed using primers TPS1-8 and TPS1-9. The amplification product, containing pBS(+) plus 0.6 kb and 0.7 kb, respectively, of flanking *tps1* sequences, was digested with *NotI* and ligated with a hygromycin resistance cassette which had been amplified from pLOB1 using primers HYG1 and HYG2 and digested with *NotI* (Doehlemann *et al.*, 2006). From the resulting plasmid, the Δ *tps1* knock-out construct was amplified with PCR, using primers TPS1-KO1 and TPS1-KO2, to yield the linear DNA which was used to transform *B. cinerea*. For disruption of *tre1*, inverse PCR was performed using primers TRE1-KO3 and TRE1-KO4, resulting in an amplification product containing pBS(+) plus 0.55 kb and 0.5 kb, respectively, of flanking *tre1* sequences. The product was digested with *NotI* and ligated with the hygromycin resistance cassette as described above. The knock-out construct was amplified from the resulting plasmid using primers TRE1-KO1 and TRE1-KO2 (Fig. 2).

Nucleic acid manipulations and sequence data analysis.

Total DNA from *B. cinerea* was isolated according to Möller *et al.* (1992). Semi-quantitative RT-PCR was performed as described by Doehlemann *et al.* (2005), using the *B. cinerea* actin gene *actA* as a constitutive control. Protein sequences were aligned by using the CLUSTAL W algorithm (Thompson *et al.*, 1994), with manual corrections at the N-termini. For creating the dendrogram, Align Plus 4 (Scientific & Educational Software) was used to perform exhaustive multi-way pairwise alignments of all sequences, using the BLOSUM62 scoring matrix, followed by progressive assembly of the alignments using the neighbour-joining method.

Measurement of intracellular trehalose in germinating conidia.

Conidia were harvested from 10-day-old tomato malt agar cultures of *B. cinerea* and washed as described by Doehlemann *et al.* (2006). A total of 10^7 conidia per experiment were used. They were germinated either in liquid HA medium [10^7 conidia (30 ml) $^{-1}$], or in Gamborg B5 minimal medium supplemented with a carbon source on glass surfaces (10^5 conidia ml $^{-1}$), or in water on polypropylene surfaces (10^5 conidia ml $^{-1}$). After incubation, the germinating conidia were harvested from the surfaces with a cell scraper. Conidia were centrifuged at 3200 g and resuspended in 100 μ l H₂O. The suspension was boiled for 5 min to eliminate enzyme activity and to release soluble sugars and polyols. After centrifugation at 11 000 g for 5 min, the supernatant was collected. Two 20 μ l aliquots of each sample were each added to 20 μ l 0.1 M sodium citrate buffer. To one of the aliquots, 4 μ l porcine kidney acid trehalase (Sigma) was added, and the samples were incubated overnight at 37 °C. The concentration of glucose in each sample was assayed photometrically with a glucose assay kit (Sigma).

RESULTS

Cloning of *Bctps1* and *Bctre1*

Based on its sequence similarity to known fungal TPSs, a *B. cinerea* EST sequence encoding parts of the *Bctps1* gene was identified. Primers complementary to the EST sequence and degenerate primers, designed according to conserved amino acid residues in TPS sequences from filamentous fungi, were used to amplify and clone a genomic *B. cinerea* fragment covering parts of the *tps1* coding region (Table 1). Later, the complete *tps1* genomic sequence was made available to us by CNRS-Genoscope, which has recently completed the *B. cinerea* genome project. The *tps1* gene (accession no.

DQ632610) contains a 1689 bp coding region, including two introns. The predicted TPS1 protein is 523 amino acids long, and has similarities of 78 and 74 % to TPS proteins from *Magnaporthe grisea* and *Aspergillus nidulans*, respectively. A sequence encoding a putative trehalose-6-phosphate phosphatase was also identified in the as yet not annotated *B. cinerea* genome, indicating that the trehalose synthesis pathway is identical to that in yeast and *A. nidulans*.

Searching the publicly accessible *B. cinerea* EST clones for trehalase sequences led to the identification of a clone encoding parts of a neutral trehalase (TRE1). A 3.4 kb DNA fragment covering the *tre1* gene was amplified from genomic *B. cinerea* B05.10 DNA, based on sequence data available from the website of the Broad Institute (http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/home.html). The 2413 bp *tre1* coding region contains four introns and encodes a predicted TRE1 protein with 727 amino acids. Sequence comparisons with other fungal trehalases revealed that TRE1 clusters with neutral trehalases from *A. nidulans* (TreB) and *N. crassa* (TreB), as well as with NTH1 from *M. grisea* (70–77 % similarity). Lower similarities (20–21 %) were found to the TRE1 trehalase of *M. grisea* and to predicted proteins from *N. crassa* and *Gibberella zeae*. A hardly detectable similarity exists between neutral trehalases and acid trehalases (Fig. 1; Amaral *et al.*, 1997).

Apart from *tre1*, the available *B. cinerea* genome sequences contain also a sequence encoding a putative acid trehalase (accession no. at Broad Institute: BC1G_12132.1). Acid trehalase has been described to be required for growth on trehalose in *A. nidulans* (d'Enfert & Fontaine, 1997). Nevertheless, on minimal medium containing 50 mM trehalose, *B. cinerea*'s growth rate reached only about

Fig. 1. Dendrogram showing the relationship of *Bc*-TRE1 (DQ632611) to other fungal trehalases. Accession numbers of the sequences shown are as follows. Neutral trehalases: *M. anisopliae* hypothetical protein (AAS67889); *N. crassa* TreB (AAC01744); *M. grisea* NTH1 (AAN46743); *A. nidulans* TreB (XP_663239); *S. cerevisiae* Nth1 (P32356); *S. cerevisiae* Nth2 (NP_009555). Mixed-type trehalases: *M. grisea* TRE1 (AAN38003); *N. crassa* hypothetical protein (XP_325123). Acid trehalases: *A. nidulans* TreA (XP_682609); *M. anisopliae* TreA (ABB51159); *B. cinerea* hypothetical protein (BC1G_12132.1); *S. cerevisiae* Ath1 (CAA58961).

20 % compared to that on other carbon sources such as glucose, fructose, sucrose and glycerol, indicating that trehalose is not a preferred carbon source of *B. cinerea* (not shown).

To monitor the activity of *tps1* and *tre1*, semi-quantitative RT-PCR experiments were performed. Similar transcript levels of both genes were found in ungerminated and germinating conidia (not shown).

Construction, growth and pathogenicity of $\Delta tps1$ and $\Delta tre1$ mutants

In order to examine the role of trehalose and trehalose mobilization in *B. cinerea*, disruption mutants of both *tps1* and *tre1* were created. The gene disruption strategies are shown in Fig. 2a, b). For both genes, three knock-out isolates ($\Delta tps1$ and $\Delta tre1$, respectively) were confirmed by PCR (Fig. 1c, d). Similar phenotypes for the knock-out isolates of each gene were observed in all experiments shown in this paper. As described below (see Fig. 4), the $\Delta tps1$ mutant contained only traces of trehalose, while increased

trehalose levels compared to the wild-type were measured in the $\Delta tre1$ mutant. This confirmed that TPS1 is required for synthesis, and TRE1 for intracellular mobilization of trehalose. Both mutants showed normal vegetative growth on various full and minimal media. Sporulation of the $\Delta tps1$ mutant reached wild-type levels, whereas the $\Delta tre1$ mutant produced only about 40 % of the number of wild-type conidia on tomato malt agar. To check the pathogenic performance of the mutants, penetration assays on onion epidermis layers (not shown) and infection tests on detached tomato leaves were performed. Both mutants showed a similar virulence to the wild-type strain (Fig. 3).

Metabolism and availability of trehalose is required for growth and heat stress tolerance

In freshly harvested wild-type conidia of *B. cinerea*, we measured a mean trehalose content of 5.2 pg, corresponding to 1.5 % of the fresh weight of a conidium. When conidial germination was induced in complete medium, rapid trehalose degradation occurred. Within 60 min, the

Fig. 2. Construction of *tps1* and *tre1* deletion mutants. (a, b) Cloning strategy for *Bctps1* and *Bctre1*. Coding regions of the disrupted genes and the hygromycin phosphotransferase gene (*hph*) are indicated by arrows. Restriction sites used for cloning are as follows: B, *Bam*HI; E, *Eco*RI; N, *Not*I; S, *Sal*I. The following primers are shown: P1, TPS(TRE)-KO1; P2, TPS(TRE)-KO2; P3, TPS(TRE)-KO3; P4, TPS(TRE)-KO4; P5, HYG-1; P6, HYG-2. (c, d) PCR-based verification of successful disruption of *Bctps1* and *Bctre1*. PCR was performed using primers P1 and P2 (as indicated above) with genomic DNA of the following *B. cinerea* strains: (c) lanes 1, wild-type; 2, $\Delta tps1$ -1; 3, $\Delta tps1$ -2; 4, $\Delta tps1$ -7; (d) lanes 1, wild-type; 2, $\Delta tre1$ -1; 3, $\Delta tre1$ -2; 4, $\Delta tre1$ -4.

Fig. 3. Infection assay with $\Delta tps1$ and $\Delta tre1$ mutants and wild-type on detached tomato leaves. At 24 and 72 h post-inoculation, no differences between mutant and wild-type strains are observed, either in primary lesion formation or in secondary lesion expansion.

trehalose content was reduced to 1.6 pg, and levelled to basal values of about 1 pg after 90 min (Fig. 4). As already described for *A. nidulans* and *N. crassa* conidia (d'Enfert & Fontaine, 1997), trehalose mobilization was observed significantly before germ tubes were formed, which did not occur until 2–3 h after addition of medium (not shown). In the $\Delta tps1$ mutant, trehalose was virtually absent in conidia, germlings and mycelium, proving that TPS1 is

Fig. 4. Changes in trehalose levels in conidia during germination in HA medium, and in response to heat shock. The arrow indicates the temperature shift from 20 to 30 °C. ■, Wild-type; ●, $\Delta tre1$ mutant; ▲, $\Delta tps1$ mutant. Values are the means $\pm$ SE of two independent experiments with at least two replicates.

required for trehalose synthesis in *B. cinerea* (Fig. 4 and data not shown). In conidia of the $\Delta tre1$ mutant, the trehalose content was increased by 20 % as compared to the wild-type. In contrast to the wild-type, no mobilization of trehalose occurred during germination in complete medium (Fig. 4). These data indicate that TRE1 is responsible for trehalose degradation during germination of *B. cinerea* conidia.

When *B. cinerea* wild-type germlings were exposed to a temperature shift from 20 to 30 °C, the trehalose content rapidly increased about tenfold. In a heat-shocked $\Delta tre1$ mutant, trehalose accumulated to even higher levels than the wild-type, whereas the $\Delta tps1$ mutant did not accumulate any trehalose (Fig. 4). At 20 °C, mycelial growth on HA complete medium was identical in all strains. At 30 °C, however, different degrees of growth inhibition were observed: radial growth of the Δtps mutant was only about 50 % compared to the wild-type. Remarkably, the $\Delta tre1$ mutant showed a slightly, but significantly, faster growth rate than the wild-type under heat stress (Fig. 5). In contrast, treatments with oxidative (H_2O_2 or paraquat) or osmotic stresses (NaCl or sorbitol) did not reveal significant differences in mycelial growth between the wild-type and the mutant strains (data not shown).

Next, it was analysed whether trehalose is also able to protect conidia against heat stress. Freshly harvested conidia of wild-type and mutant strains were exposed to 45 °C for different times, and their viability was subsequently tested by monitoring their germination rate on HA agar. As expected, $\Delta tps1$ conidia were less able to survive heat stress, and lost viability more rapidly during heat treatment than wild-type conidia. In contrast, the heat tolerance of $\Delta tre1$ conidia was not significantly different from that of wild-type conidia (Fig. 6). In a TPS-deficient mutant of *A. nidulans*, conidia had a reduced viability during storage (Fillinger *et al.*, 2001). A similar phenotype was observed for the $\Delta tps1$ mutant. After 2 weeks storage at 20 °C, the $\Delta tps1$ conidia had almost completely lost their viability, while wild-type conidia still showed a germination rate of about 85 % (Fig. 7b). Microscopic investigation revealed that 2-week-old $\Delta tps1$ conidia were severely affected in their cell integrity. After suspension in water, aggregation of the cytoplasm was observed in the majority of spores (data not shown). After treatment with 0.1 % Tween 20, most $\Delta tps1$ conidia were found to be collapsed whereas wild-type spores remained unaffected by this treatment (Fig. 7a). These findings strongly support the role of trehalose as a protective agent increasing the survival of conidia at both moderate and high temperatures.

The role of trehalose metabolism in conidial germination

The following experiments were performed to analyse trehalose metabolism and to clarify its role during germination. As already shown in Fig. 4, rapid mobilization of trehalose occurred in conidia germinated in full medium. When germination was induced by 10 mM fructose on a

Fig. 5. Growth tests at 20 °C and 30 °C with *B. cinerea* wild-type, $\Delta tps1$ and $\Delta tre1$ mutants on HA agar plates. (a) Colony morphology after 7 days of growth at 30 °C. (b) Colony expansion rates at 20 °C and 30 °C. ■, wild-type; ●, $\Delta tre1$ mutant; ▲, $\Delta tps1$ mutant. Values are the means $\pm$ SE of two independent experiments with at least three replicates.

glass surface, trehalose was also quickly degraded prior to visible germination. After 2 h, trehalose concentration had dropped to approximately 20 % of the concentration in ungerminated conidia, and it stayed at this level during germ tube outgrowth. In contrast, spores that had germinated on a hydrophobic surface in water showed a very delayed trehalose breakdown. During the first 4 h, trehalose did not seem to be degraded at all. After 8 h, when 20 % of the conidia had germinated, the trehalose contents had decreased by only about 30 %, whereas at the same germination rate in fructose-supplemented medium the trehalose concentration had already reached its lowest level (Fig. 8). In yeast and *A. nidulans*, neutral trehalases are known to be activated by

cAMP (d'Enfert *et al.*, 1999; Amaral *et al.*, 1997; de Almeida *et al.*, 1997). In *B. cinerea*, however, cAMP was found to be involved only in carbon-source-induced germination, but not in germination on hydrophobic surfaces (Doehlemann *et al.*, 2006). We therefore reasoned that TRE1 is not activated during germination in the absence of nutrients due to the lack of cAMP. To test this idea, wild-type conidia were germinated on a polypropylene surface in water, supplemented with the membrane-permeable cAMP derivative CPT-cAMP. Indeed, this treatment resulted in a more rapid mobilization of trehalose, concomitantly with a strongly accelerated germination (Fig. 8).

$\Delta tre1$ and $\Delta tps1$ mutants show specific germination defects

To further investigate the importance of trehalose in germinating conidia, we compared the germination behaviour of the $\Delta tre1$ and $\Delta tps1$ mutants and the wild-type. In full medium, rapid germination of conidia occurred, with no difference between the wild-type and the mutants (data not shown). When conidia were germinated in minimal medium containing low amounts of sugars, clear differences between the strains were evident. In the presence of 10 mM glucose or fructose, the $\Delta tre1$ mutant showed a slightly delayed germination when compared to the wild-type (Fig. 9a). This delay was more pronounced in the presence of only 1 mM glucose or fructose; nevertheless, after 24 h, almost full germination of $\Delta tre1$ conidia had occurred (Fig. 9b). Since carbon-source-induced germination was found to be mediated by cAMP-dependent signalling (Doehlemann *et al.*, 2006), these data are in agreement with the stimulating effect of cAMP on trehalose mobilization and germination of wild-type conidia on hydrophobic surfaces. Our data also indicate that trehalose mobilization accelerates germination at low external nutrient concentrations. When conidia were germinated on a hydrophobic

Fig. 6. Survival of *B. cinerea* conidia after heat shock treatment. Wild-type (■), $\Delta tre1$ (●) and $\Delta tps1$ (▲) mutants were incubated at 45 °C for the indicated times. Subsequently, the germination rate of the conidia was determined after 24 h incubation on glass slides with HA agar. Values are the means $\pm$ SE of two independent experiments with at least two replicates.

Fig. 7. Loss of viability of $\Delta tps1$ conidia after prolonged storage at 20 °C. (a) Micrographs of conidia after 15 days storage and suspension in 0.1 % Tween 20. Scale bars, 10 μ m. (b) Viability of dry conidia after different times of incubation at 20 °C, tested by their germination rate after 24 h incubation on glass slides with HA agar. ■, Wild-type; ●, $\Delta tps1$ mutant. Values are means $\pm$ SE of two independent experiments with at least three replicates.

surface in the absence of external nutrients (conditions that do not induce trehalose mobilization; cf. Fig. 8), both $\Delta tre1$ and $\Delta tps1$ mutants showed germination kinetics similar to the wild-type (Fig. 9c).

Remarkably, germination in the presence of 10 mM glucose or fructose was more strongly reduced in $\Delta tps1$ conidia (Fig. 9d). This phenotype cannot be explained just by the lack of trehalose as an energy source, considering the comparatively minor germination defect of the $\Delta tre1$ mutant. Yeast and *M. grisea* mutants lacking TPS have been described to be seriously handicapped in their growth

on glucose or fructose as sole carbon sources (Bell *et al.*, 1998; Foster *et al.*, 2003). This was explained by the lack of trehalose 6-phosphate (T6P) which acts as a negative regulator of hexokinase activity and which is required for the control of glycolysis (Thevelein & Hohmann, 1995; Bell *et al.*, 1998; Foster *et al.*, 2003). We did not observe any defect in mycelial growth of the $\Delta tps1$ mutant in minimal media containing glucose or fructose. Nevertheless, germination of the $\Delta tps1$ mutant in the presence of galactose (which is phosphorylated in the cell not by hexokinase but by galactokinase) was similar to the wild-type germination after 24 h (Fig. 9d). We therefore suggest that the germination defect of the $\Delta tps1$ mutant in the presence of glucose or fructose is mainly due to the lack of T6P as a regulator of carbohydrate metabolism.

Fig. 8. Trehalose breakdown during conidial germination. Trehalose contents were measured from conidia germinating on glass in Gamborg B5 medium supplemented with 10 mM fructose (□), on polypropylene in water (○), and on polypropylene in water supplemented with CPT-cAMP (100 μ g ml⁻¹; △). The corresponding germination rates are indicated with solid symbols. Values are the means $\pm$ SE of two independent experiments with at least two replicates.

DISCUSSION

In this work, we have analysed the role of trehalose and its metabolism in *B. cinerea*.

Our results indicate an important role of trehalose as a protectant concerning both heat stress tolerance and general conidial survival. Trehalose metabolism plays a role in conidial germination in two aspects: first, trehalose provides a significant source of energy for the germinating spore when external nutrient sources are limiting. Second, the trehalose precursor T6P seems to play a role in regulation of the glycolytic flux, which is also important for induction of germination.

To analyse the role of trehalose synthesis in *B. cinerea*, mutants defective in TPS and a neutral trehalase were created. The absence of trehalose from $\Delta tps1$ conidia confirmed that TPS is essential for trehalose synthesis. The phenotype of the $\Delta tre1$ mutant showed that the neutral trehalase TRE1 is required for breakdown of intracellular trehalose, similar to the situation in other fungi.

Fig. 9. Germination rates of *B. cinerea* conidia under different conditions. (a, b) Wild-type (■) and $\Delta tre1$ mutant (●) in Gamborg B5 medium with 10 mM fructose (a) or with 1 mM fructose (b), on glass. Note that the *x*-axis scales are not linear. (c) Wild-type (■), $\Delta tre1$ mutant (●) and $\Delta tps1$ mutant (▲) in water, on polypropylene. (d) Wild-type (black bars) and $\Delta tps1$ mutant (grey bars) in Gamborg B5 with different sugars, on glass, 24 h post-inoculation. Black bars, wild-type; grey bars, $\Delta tps1$. Values are the means $\pm$ SE of two independent experiments with at least three replicates.

Regarding their growth phenotype, both mutants showed no difference from the wild-type when growing on minimal or full media, except for a moderate reduction of sporulation efficiency of the $\Delta tre1$ mutant. Disruption of the neutral trehalase led also to reduced sporulation in *M. grisea* but not in *A. nidulans* (Foster *et al.*, 2003; d'Enfert *et al.*, 1999). Similar to *B. cinerea*, a TPS-deficient mutant of *A. nidulans* showed normal vegetative growth (Fillinger *et al.*, 2001). In contrast, TPS mutants of yeast and *M. grisea* were unable to grow in minimal media containing glucose or fructose (Foster *et al.*, 2003; Thevelein & Hohmann, 1995). This was explained by the absence of T6P and will be discussed further below.

In the rice blast pathogen *M. grisea*, TPS-deficient mutants did not synthesize trehalose and formed appressoria which were defective in penetration, whereas mutants defective in the neutral trehalase NTH1 showed successful penetration but a decreased ability to colonize plant tissue (Foster *et al.*, 2003). In contrast, neither $\Delta tps1$ nor $\Delta tre1$ mutants of *B. cinerea* showed any defects in pathogenicity. The turgor pressure in the morphologically often poorly differentiated appressoria of *B. cinerea* is unknown, but probably much lower than the pressure in mature melanized appressoria of

M. grisea, which has been estimated to be up to 80 bar (de Jong *et al.*, 1997). Thus, in *B. cinerea* germlings, the mobilization of trehalose does not seem to be essential for subsequent plant cell penetration.

As in other fungi, trehalose was found to be an important heat protectant in *B. cinerea*. After heat-shock treatment, the trehalose concentration in wild-type germlings quickly increased almost tenfold, to about twofold storage levels of conidia. Growth of the $\Delta tps1$ mutant was much more inhibited than that of the wild-type at 30 °C. Interestingly, the $\Delta tre1$ mutant was found to be more heat resistant than the wild-type, showing about 20 % increase in radial growth, presumably due to its increased trehalose levels. Protection by trehalose was also evident in the resting stage of *B. cinerea*: survival of $\Delta tps1$ conidia at 45 °C was lowered, with a half-life reduction of about 25 % compared to the wild-type and the $\Delta tre1$ mutant. Furthermore, $\Delta tps1$ conidia displayed a drastically reduced survival at normal temperature. In *A. nidulans*, lack of trehalose due to disruption of the *tpsA* gene also led to increased sensitivity of growth to high temperatures as well as oxidative stress, and also to a rapid loss of viability of conidia at ambient temperature. However, *tpsA* conidia showed similar sensitivity to heat

treatment as wild-type conidia (Fillinger *et al.*, 2001). No data regarding heat stress tolerance have been reported for trehalose-deficient mutants in *M. grisea* (Foster *et al.*, 2003). In baker's yeast, trehalose has also been described to contribute to osmotic stress tolerance (Hounsa *et al.*, 1998). In contrast, we did not find evidence for this in *B. cinerea*, nor for its role in protection against other stress factors. Thus, trehalose appears to play similar but not identical roles in cellular protection in different fungi.

Substantial amounts of trehalose have been found in conidia of various fungi. For instance, *N. crassa* and *A. nidulans* contain up to 10 and 4% trehalose (per fresh weight), respectively (Schmitt & Brody, 1976; Ruijter *et al.*, 2003). The trehalose content of *B. cinerea* conidia was estimated to be about 1.5%. Trehalose breakdown has been described in *A. nidulans* and *N. crassa* as the first detectable biochemical event following germination induction, occurring much earlier than germ tube appearance (d'Enfert, 1997). Similarly, in *B. cinerea* conidia which were induced to germinate in the presence of full media or 10 mM fructose, trehalose degradation started to occur after 30 min, while germination was not observed until after 3–4 h. In contrast, trehalose degradation was strongly delayed and did not precede germination in conidia that were induced to germinate on a hydrophobic surface, in the absence of nutrients. However, addition of cAMP to these conidia resulted in significant mobilization of trehalose and accelerated germination. In *Saccharomyces cerevisiae*, trehalose breakdown requires activation of the neutral trehalase, mediated by cAMP-dependent protein kinase A and possibly Ca^{2+} (Souza *et al.*, 2002; d'Enfert 1997; de Almeida *et al.*, 1997). The inability of adenylate-cyclase-deficient *N. crassa* mutants to mobilize trehalose during germination also indicates that cAMP signalling is required for activation of neutral trehalase (de Pinho *et al.*, 2001). We therefore hypothesize that TRE1 activity in *B. cinerea* is dependent on cAMP signalling as well. Further support for this came from the observation that a *B. cinerea* Δbcg3 mutant, which is defective in cAMP signalling, does not show any degradation of trehalose during germination (Doehlemaun *et al.*, 2006; data not shown). Similarly, it has been demonstrated recently that G-protein-mediated cAMP signalling, activated by hexoses, is necessary for trehalase activity in *A. nidulans* (Lafon *et al.*, 2005). This is supported by the fact that the predicted TRE1 protein contains the typical N-terminal RRXS motive, which has been described as a cAMP-dependent protein kinase phosphorylation site (Kopp *et al.*, 1993; Rittenhouse *et al.*, 1986). Other typical features such as a Ca^{2+} -binding domain are also conserved in the TRE1 sequence (Amaral *et al.*, 1997).

The rapid mobilization of trehalose in germinating conidia indicated that it is an important energy source for germination. *A. nidulans* conidia lacking the neutral trehalase TreB showed a delay in germination when incubated under carbon limitation (d'Enfert *et al.*, 1999). The germination phenotype of the *B. cinerea* mutants was

found to be dependent on the signal which induced germination. Both mutants germinated in a similar way to the wild-type in rich media or on hydrophobic surfaces in water. However, a delayed germination was observed for the *B. cinerea* Δtre1 mutant in the presence of sugars at low concentrations, suggesting an energetic role of trehalose in the initial phase of germination. An even stronger germination defect was observed in the presence of glucose or fructose in the Δtps1 mutant. This was different from *A. nidulans* and *M. grisea* TPS mutants, which were not reported to be defective in germination (Fillinger *et al.*, 2001; Foster *et al.*, 2003). On the other hand, disruption of TPS activity in *M. grisea* resulted in the inhibition of growth on several carbon sources, similar to what has been described for yeast (Thevelein & Hohmann, 1995). This growth disturbance of TPS-deficient mutants has been explained by the deregulation of glycolytic flux due to the absence of T6P (Gancedo & Flores, 2004; Eastmond & Graham, 2003; Thevelein, 1996). A similar explanation for the impaired germination of the Δtps1 mutant in the presence of fructose or glucose was supported by the observation of a high sensitivity of *B. cinerea* hexokinase to T6P. In extracts prepared from a *B. cinerea* mutant expressing only hexokinase but not glucokinase activity, addition of 1 mM T6P resulted in 90% reduction of hexose phosphorylation activity (O. Rui & M. Hahn, unpublished). This strongly supports the idea that glycolytic deregulation in the Δtps1 mutant disturbs germination in the presence of glucose or fructose, but not with galactose.

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