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Research

Trehalose turnover during abiotic stress in arbuscular mycorrhizal fungi

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Summary

• Trehalose is a common reserve carbohydrate in fungi, whose role has been recently extended to other cellular functions, such as stress tolerance, glycolysis control, sporulation and infectivity of some pathogenic strains.

• To gain some insight into the role of trehalose during abiotic stress in arbuscular mycorrhizal (AM) fungi, we assessed trehalose content as well as transcriptional regulation and enzyme activity of neutral trehalase and trehalose-6-phosphate phosphatase in *Glomus intraradices* in response to heat shock, chemical or osmotic stress.

• Prolonged or intensive exposure to heat or chemical stress, but not osmotic stress, caused an increase of trehalose in the cell. We found this associated with transient up-regulation of the trehalose-6-P phosphatase (*GiTPS2*) transcript that coincided with moderate increases in enzyme activity. By contrast, there were no changes in neutral trehalase (*GiNTH1*) RNA accumulation in response to stress treatments, while they promoted, in most cases, an increase in activity. After stress had ceased, trehalose returned to basal concentrations, pointing to a role of neutral trehalase activity in heat shock recovery. A yeast complementation assay confirmed the role of neutral trehalase in thermotolerance.

• Taken together, these results indicate that trehalose could play a role in AM fungi during the recovery from certain stresses such as heat shock and chemical treatment.

Key words: abiotic stress, *Glomus intraradices*, *Glomus mosseae*, mycorrhizal fungi, neutral trehalase, trehalose, trehalose-6-P phosphatase.

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Introduction

Microorganisms have evolved different strategies to cope with environmental stresses. Stress induces a cellular stage that usually leads to the synthesis of specific proteins and metabolites in order to adapt and survive until better conditions return. Heat-shock proteins or hydrophilins are a good example, conserved in evolution, of common cell response against hostile environmental conditions (Garay-Arroyo *et al.*, 2000; Hohmann, 2002). A common response of organisms to drought, salinity, and low-temperature stresses is the accumulation of sugars and other compatible solutes as osmoprotectants that act by stabilizing biomolecules (Voit, 2003). One such compound is trehalose (α-D-glucopyranosyl, α-D-glucopyranoside), a nonreducing disaccharide, consisting of two molecules of glucose bound by an α,α-1,1-glycosidic linkage. Trehalose is also present in bacteria, eukaryotic microorganisms, plants, insects and invertebrates, but up to now has not been found in vertebrates (Benaroudj *et al.*, 2001). In all organisms where trehalose is present, it has been found to play an important

physiological role as protectant against abiotic stress (Crowe *et al.*, 1992). Trehalose accumulation to protect the cell is likely to be an evolutionarily old mechanism, since even Archaebacteria accumulate trehalose in response to stress (Nicolaus *et al.*, 1988). Trehalose protects the cells by stabilizing cell structures and enables proteins to maintain their native conformation under stress conditions (Singer & Lindquist, 1998; Eroglu *et al.*, 2000). Interestingly, the presence of trehalose interferes with protein refolding, and that explains why trehalose is quickly degraded after stress has ceased (Singer & Lindquist, 1998). Trehalose is commonly found in fungi and at particularly high concentrations in resting cells and survival forms, such as spores and sclerotia. In addition to its major function as carbohydrate reserve, and similarly to other organisms, in fungi trehalose also protects against several adverse conditions such as heat, desiccation, freezing (van Laere, 1989), hydrostatic pressure (Iwahashi *et al.*, 2000), nutrient starvation, and several abiotic stresses such as osmotic, oxidative or chemical stresses (Zähringer *et al.*, 2000). In arbuscular mycorrhizal (AM) fungi, trehalose and glycogen are the main storage carbohydrates (Bécard *et al.*, 1991). However, these fungi preferentially accumulate carbon (C) in the form of triacylglycerols (TAGs) (up to 95%) during their life cycle. AM fungi obtain the C through the symbiosis with their partner, the plant. Carbon is drawn from the root cortex, by the so-called intraradical mycelium in the form of hexoses and transformed quickly to trehalose and glycogen, and further to lipids (Shachar-Hill *et al.*, 1995; Pfeffer *et al.*, 1999). Translocation of C to the distal part of the fungal colony, the extraradical mycelium, has been shown to take place mainly in the form of glycogen and TAGs (Bago *et al.*, 2003). This extraradical mycelium spreading into the soil is responsible for the mineral nutrient uptake that benefits its plant host, and ultimately produces the asexual propagules, the chlamydospores, that complete the life cycle. Trehalose has been shown to be present in extraradical mycelium as well as in spores of AM fungi, and in all cases it has been attributed a mere role as intermediate C storage (Bécard *et al.*, 1991; Bago *et al.*, 2002, 2003). Although there are reports showing the accumulation of trehalose in mycorrhizal plants in response to drought stress (Schellenbaum *et al.*, 1998, 1999), the possible role of trehalose as cellular stress protectant in extraradical mycelium has not been investigated.

In yeast and other fungi, trehalose synthesis takes place in a two-step process catalyzed by the trehalose synthase enzyme complex comprising four subunits (Bell *et al.*, 1992; de Virgilio *et al.*, 1993; Vuorio *et al.*, 1993; Reinders *et al.*, 1997). In the first step, a glucosyl residue is transferred from uridine-5-diphosphoglucose (UDPG) to glucose-6-phosphate to form trehalose-6-phosphate. This reaction is catalyzed by the trehalose-6-phosphate synthase subunit (TPS1). In a second step, the phosphate group is removed from trehalose-6-phosphate to render trehalose and orthophosphate. This step is catalyzed by a second enzyme, the trehalose-6-phosphate phosphatase (TPS2). Two other subunits without catalytic activity have been shown to form part of the complex and to participate in the regulation of the trehalose synthesis, TPS3 and TSL1. Investigations in other fungi, including the filamentous fungus *A. nidulans* and *A. niger*, have shown that they synthesize trehalose using a an enzymatic complex that includes at least TPS1 and TPS2 (Borgia *et al.*, 1996; Wolschek & Kubicek, 1997). Expression of TPS1, TPS2, and TSL1 in yeast is strongly stimulated by all stress conditions, while TPS3 expression is only weakly stimulated by heat shock (Winderickx *et al.*, 1996; Gasch *et al.*, 2000; Causton *et al.*, 2001). Mutants for TPS1 and TPS2 are unable to accumulate trehalose and display a low thermotolerance phenotype. None of the genes responsible for trehalose synthesis and their encoding enzymes has been isolated so far in AM fungi.

The hydrolysis of trehalose into two molecules of glucose is performed by trehalases (Jorge *et al.*, 1997). On the basis of catalytic properties, subcellular localization and mechanisms of regulation, trehalases have been grouped into two classes: acid trehalases, extracellular and vacuolar glycoproteins, permanently active, with a high thermal stability and an optimum pH of approx. 4.5 (Wiemken & Schellenberg, 1982; Londesborough & Varimo, 1984); and neutral trehalases, cytosolic proteins with an optimum pH of approx. 7.0, a much lower thermal stability and a lower substrate affinity (Thevelein, 1984). Acid and neutral trehalases have specific and independent roles. They are distinct types of enzymes, which only share a strict specificity for trehalose. Neutral trehalases are considered key enzymes responsible for the internal trehalose breakdown and they are tightly regulated enzymes. Interestingly, it was shown that exposure to different types of stresses also induced neutral trehalase concomitant to the induction of enzymes of trehalose biosynthesis in different fungi (Zähringer *et al.*, 1997, 2000; d'Enfert *et al.*, 1999). Although in principle paradoxical with the concept of trehalose accumulation during stress, the current hypothesis suggests that neutral trehalase activation might be important for recovery after stress by inducing trehalose mobilization. Consistent with this hypothesis, neutral trehalase mutation produces in yeast the so-called 'poor heat shock recovery phenotype'. These mutants, in contrast to the wild-type, are impaired in growth at normal temperature after exposure for some hours to sublethal heat shock (Nwaka *et al.*, 1995). In AM fungi, trehalase activity with an optimum pH of *c.* 7 was reported in germinated sporocarps and extraradical mycelium (Schubert *et al.*, 1992; Schubert & Wyss, 1995). However, no further experiments have been carried out.

To investigate in more detail the role of trehalose in AM fungi, we have studied the gene expression and the activity of two key enzymes in the trehalose metabolism, neutral trehalase (trehalose mobilization) and trehalose-6-phosphate phosphatase (trehalose synthesis), and correlated this with trehalose concentrations in the hyphae. This study is aimed, in particular, at ascertaining whether trehalose turnover plays a role during abiotic stress in AM fungi.

Table 1 *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Derived from:	Reference
T19	Prototroph (*MATα NTH1*)		Shima *et al*. (1999)
T19dN	*MATα nth1::URA3*	T19	Shima *et al*. (1999)
T19-kanMX4	*MATα ura3*::kanMX4	T19	This work
T19dN-kanMX4	*MATα nth1::URA3 ura3*::kanMX4	T19dN	This work
T19dN-kanMX4-GmNTH1	*MATα nth1::URA3 ura3*::kanMX4 Plasmid pAO196*GmNTH1*	T19dN::kanMX4	This work

Materials and Methods

Fungal strains and growth conditions

The AM fungus *Glomus intraradices* was cultivated in the *in vitro* system described by Bécard & Fortin (1988). Ri T-DNA-transformed carrot (*Daucus carota* L) roots colonized with *G. intraradices* Schenck and Smith (DAOM 181602) were grown in bicompartmental Petri plates as described by St-Arnaud *et al*. (1996) at 27°C in the dark. Carrot roots were grown in the distal compartment of the divided plate containing solid M medium (Bécard & Fortin, 1988) supplemented with 1% sucrose at pH 5.5. Only hyphae were allowed to grow in the proximal compartment containing solid or liquid M medium without sucrose. For hyphal growth in liquid medium, solid medium was removed, once the fungus reached the second compartment, and refilled with 8 ml liquid M medium (Bécard & Fortin, 1988). Sporocarps from *G. mosseae* (Nicol & Gerd.) Gerdermann & Trappe (BEG12) were produced using parsley as a host plant. They were surface-sterilized following the method described by Budi *et al*. (1999). Sporocarps were germinated on water-agar plates as described by Requena *et al*. (1999).

Saccharomyces cerevisiae strains used are listed in Table 1. Yeasts were routinely grown in YPDA medium at 30°C for 2–3 d.

Abiotic stress treatments in *G. intraradices* cultures

G. intraradices extraradical hyphae grown for 10 d (exponential growth phase) in liquid M medium were subjected to heat, osmotic and chemical stress. For heat stress, temperature of growth was elevated to 37°C and cultures were exposed to this temperature for 1, 2 or 5 h. For osmotic stress, salt concentration was increased to 0.5 M of NaCl. Samples were collected after 12 and 24 h. For chemical stress, 1 mM disodium arsenate was used. Hyphae were exposed to arsenate for 20 and 40 min. Control treatments in all cases corresponded to hyphae prepared in the same way, but without the stressors, and incubated for the longest period corresponding to each treatment (5 h, 24 h and 40 min, respectively).

Determination of trehalose content

Trehalose content in *G. intraradices* hyphae was measured by the acid trehalase method (Winkler *et al*., 1991). Briefly, hyphae were recovered from the liquid medium using forceps and immediately frozen in liquid nitrogen. They were disrupted by mortar and pestle under liquid nitrogen and resuspended in 50 µl distilled water. Trehalose was extracted by 20 min boiling in a water bath. Six microliters of each sample were analyzed with 6.5 µl of 200 mM citrate/NaOH buffer, pH 5.7, using 2.5 µl of porcine kidney trehalase (2.5 mU; Sigma, Taufkirchen, Germany). After incubation at 37°C for 1 h, the reaction was stopped in a boiling water bath for 5 min. Glucose liberated was determined by the hexokinase/glucose-6-P dehydrogenase method (Einig & Hampp, 1990). Briefly, samples were assayed in a 100 µl reaction mixture containing 17.6 mM Tris-HCl, pH 7.0, 1.6 mM Mg^{2+}, 0.25 mM ATP, 0.25 mM NADP, 0.2 U ml^{-1} glucose-6PDH, 1.4 U ml^{-1} hexokinase). Values were corrected by subtracting sample glucose, assayed before addition of acid trehalase. Absorbance was measured at 340 nm.

Protein extracts and enzyme activities

Hyphae were collected and disrupted using mortar and pestle under liquid nitrogen. The ground material was resuspended in 350 µl of extraction buffer (50 mM Tris-HCl, pH 7.5, 75 mM NaCl, 7 mM β-mercaptoethanol, 50 mg ml^{-1} PVPP (polyvinylpolypyrrolidone) 10.000, 4 µg ml^{-1} chymostatin) and placed on ice for 10 min. The homogenized mixture was centrifuged at 10 000 ***g*** at 4°C for 10 min. The supernatant was recovered and frozen at −20°C until use. Protein concentration was determined with the Bio-Rad protein assay (Bio-Rad, Munich, Germany).

Determination of trehalose-6-P phosphatase activity

Trehalose-6-P phosphatase activity was measured in *G. intraradices* hyphae as described by de Virgilio *et al*. (1992). Protein extract (50 µl) was assayed in a 100 µl reaction mixture containing 1.6 mM trehalose-6-phosphate (Sigma),

10 mM $MgCl_2$, 25 mM (Na^+/K^+) phosphate buffer, pH 6.0. After incubation at 30°C for 20 min, the reaction was stopped in a boiling water bath for 5 min. The trehalose formed was measured as already described.

Determination of neutral trehalase activity

In order to determine neutral trehalase activity, the enzyme was activated by phosphorylation via cAMP (cyclic adenosine monophosphate). Five microliters of the protein extract were incubated at 30°C for 30 min with the activation mix (3.6 mM ATP, 0.18 mM cAMP (for activation of sample PKA, protein kinase A), 3.6 mM $MgCl_2$ in 50 mM imidazole-HCl, pH 7.0, in a final volume of 10 µl). After this time, neutral trehalase activity was measured at 37°C in 100 µl of a reaction mix containing 130 mM Tris-HCl, pH 7.5, and 250 mM trehalose, by the determination of glucose (Einig & Hampp, 1990).

All enzymatic measurements were carried out in triplicate and the experiment was conducted twice, sometimes three times. Error bars correspond to the standard deviation.

Gene expression studies by RT-PCR

Gene expression studies were carried out for the genes encoding trehalose-6-P phosphatase and neutral trehalase in *G. intraradices* by RT-PCR (reverse transcriptase- polymerase chain reaction) as described previously (Breuninger & Requena, 2004; Breuninger *et al.*, 2004). RNA was extracted from extraradical hyphae after homogenization in liquid nitrogen using the TRIZOL method according to manufacturer's instructions (Invitrogen, Karlsruhe, Germany). Specific primers GiTPS2F2 (5′-GAACTGCTATAATGGTAAATCCTTGG-3′) and GiTPS2R2 (5′-GATCGGTGTTAATGTTCCAT-CATAATC-3′) were used to study trehalose-6-phosphate synthase at 57°C annealing temperature during 35 cycles. GiNTF3 (5′-CCGCTCTTTGGGCACTTTGG-3′) and GiNTR2 (5′-TTCATCCAACCGAAACCCTC-3′) were used to study neutral trehalase expression at 53°C annealing temperature in a 30-cycle PCR. Results were obtained in triplicate and pictures shown are representative of the results obtained.

Heat shock recovery assay in extraradical hyphae

To determine whether the concentrations of trehalose returned back to basal values after heat shock treatment, *G. intraradices* extraradical hyphae grown for 10 d in liquid M medium were subjected to heat stress, increasing the temperature of growth to 37°C. Samples were taken at 1, 2 and 5 h. After this time plates were returned to normal growth temperature and hyphae allowed to grow for further 24 h. At that time, stressed and control samples were again harvested and the content of trehalose analyzed.

Heterologous complementation of yeast neutral trehalase mutants

The *G. intraradices* cDNA encoding neutral trehalase was used as heterologous probe to screen a cDNA expression library of the AM fungus *Glomus mosseae* (Ocón, 2004). A clone containing the full-length cDNA of the *G. mosseae* neutral trehalase was isolated. The neutral trehalase open reading frame (ORF) was amplified by PCR using the primers GmNT5end′ 5′-GAAATGACAGACACAGAAGC-3′ and GmNT3end′ 5′-GATCAACCAATTGAACTTTCTTG-3′. Amplification was carried out with the High Fidelity Expand Polymerase (Roche, Penzberg, Germany). The amplified product was cloned in TOPO PCR2.1 vector (Invitrogen). *GmNTH1* was *Spe*I-*Xho*I subcloned into the yeast expression vector pDR196 containing the URA3 marker, under the control of the constitutive yeast H^+-ATPase promoter, PMA1, and the alcohol dehydrogenase (ADH1) terminator (D. Rentsch, personal donation). Neutral trehalase yeast mutant strain (T19dN) and its parental wild-type (T19) (Shima *et al.*, 1999) are prototroph for uracil. In order to create *URA3* mutants of these strains, the *URA3* gene was disrupted using a PCR-based strategy. A module containing the Geneticin resistance cassette (*kanMX4*) flanked by the upstream and downstream regions of the *URA3* gene from *S. cerervisiae* was constructed by PCR using the plasmid pFA6a-KanMX4 as template. This module was directly used for yeast transformation. Selection of transformants was performed in plates containing uracil and geneticin as previously described (Knop *et al.*, 1999). Transformation of neutral trehalase yeast mutants was carried out using the LiAc method (Gietz & Woods, 2002).

Heat shock recovery assay in yeast

Transformed yeast containing the neutral trehalase gene of *G. mosseae*, the neutral trehalase mutants and their corresponding wild-type strains were streaked out on YPDA complete solid media and grown for 2 d at 30°C. They were then replica-plated using a velvet stamp in fresh YPDA media and immediately incubated at 50°C for 6 h. After this time, plates were further incubated for 2 d at 30°C.

All sequences from this paper have been deposited at the NCBI database under the accession numbers AY787133–AY787135 and AY830086.

Results

Isolation of *GiTPS2*, *GmTPS2*, *GiNTH1* and *GmNTH1*

In order to isolate the genes encoding trehalose-6-P phosphatase and neutral trehalase from AM fungi, we used a PCR approach with degenerated primers on cDNA from spores of *G. intraradices*. Primers for neutral trehalase were NTF1 5′-CATGATRGWGCWGTWMG-3′ and NTR1

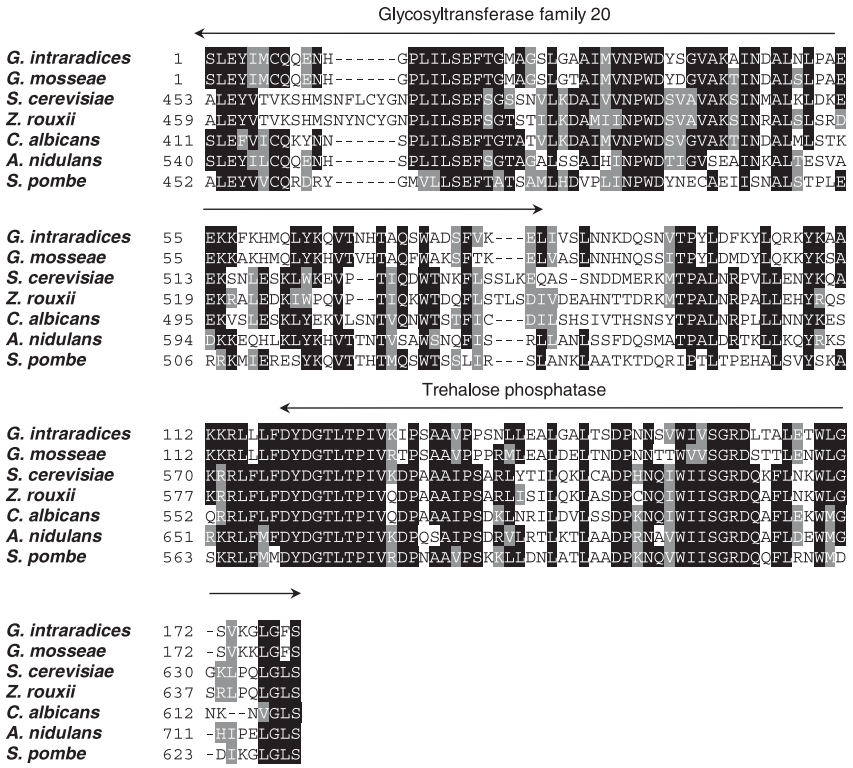

Fig. 1 Alignment for maximal amino acid similarity of the partial *Glomus intraradices* and *G. mosseae* TPS2 sequences with *Aspergillus nidulans* (AAO72737), *Saccharomyces cerevisiae* (CAA98893), *Zygosaccharomyces rouxii* (AAF80562), *Candida albicans* (CAC17748) and *Schizosaccharomyces pombe* (P78875) trehalose-6-phosphate phosphatases. Conserved domains with proteins of the glycosyltransferase family 20 and trehalose phosphatases (EC:3.1.3.12) are indicated. Alignment was done with the program CLUSTAL X and displayed using the online program BOXSHADE 3.2.1. Identical amino acids are shaded in black, conserved amino acids in grey.

5′-WGGRTAATCCCAYTG-3′; and for trehalose-6-P TPS2F1 5′-TCCCTTGARTAYATHATWTG-3′ and TPS2R1 5′-SWNARWCCWARWCC-3′. Two DNA fragments (545 and 536 bp) putatively encoding neutral trehalase and trehalose-6-P phosphatase were isolated. The genes were designated *GiTPS2* and *GiNTH1*, respectively. BLASTX analysis of *GiTPS2* sequence fragment showed 53% sequence identity with OrlA, the trehalose phosphate phosphatase from the filamentous fungus *A. nidulans*. A corresponding sequence (680 bp) from the AM fungus *G. mosseae* (*GmTPS2*) was isolated by screening of an ESTs library (M. Breuninger & N. Requena, unpublished), using the *G. intraradices* fragment as a probe. The deduced GiTPS2 and GmTPS2 proteins contain the conserved domain characteristic of trehalose phosphatases (EC:3.1.3.12) that catalyze the dephosphorylation of trehalose-6-phosphate to trehalose and orthophosphate, and the conserved domain of the gylcosyltransferase family 20 (Fig. 1). Full-length cDNA of GiNTH1 was isolated using the RACE protocol from GeneRacer (Invitrogen) according to manufacturer's instructions. A 2943 bp cDNA was sequenced revealing an ORF of 2223 bp encoding a predicted protein of 741 amino acids with a calculated mass of 86 kDa. A partial cDNA clone (777 bp) of a neutral trehalase from *G. intraradices* was found in the NCBI database (BI246186); the clone has full identity to our sequence and therefore we assume they encode the same protein (Jun *et al.*, 2002). The full-length GmNTH1 was isolated by screening a cDNA library (SMART cDNA library, A. Ocón and N. Requena, unpublished) using the partial neutral trehalase cDNA sequence from *G. intraradices* as a probe. Sequencing of the clone revealed an ORF of 2346 bp encoding a predicted protein of 781 amino acids with a calculated molecular mass of 89.7 kDa. The deduced amino acid sequence of GiNTH1 and GmNTH1 proteins showed 82% similarity among each other and also showed similarity to neutral trehalase sequences from other fungi such as *Ustilago maydis* (61%), *Crytococcus neoformans* (61%), *S. pombe* (59%), *Neurospora crassa* (56%), *Metarhizium anisopliae* (55%), and *Magnaporthe grisea* (55%).

Prosite analysis of the deduced neutral trehalases sequences showed the presence of the trehalase signatures 1 and 2, with consensus sequences P-G-G-R-F-x-E-x-Y-x-W-D-x-Y and Q-W-D-x-P-x-[GA]-W-[PAS]-P, respectively (Fig. 2). This signature is specific to trehalases and is not present in any other protein. Both proteins contained also a cAMP-dependent protein kinase phosphorylation site, RRaS, with a conserved phosphorylatable serine (Fig. 2). A motif highly similar to EF-like Ca^{2+}-binding sites was localized at the N-terminus of both proteins (Fig. 2). No signal peptides were identified in the sequences, suggesting that GmNTH1 and GiNTH1 are probably intracellular protein, as has been demonstrated for other neutral trehalases (Xia *et al.*, 2002). Interestingly, GiNTH1 contained a peroxisomal targeting signal (SKF) at the carboxy-terminus, absent in its ortholog in *G. mosseae*. Both proteins were very divergent at this end, despite the high degree of similarity throughout the whole length of the protein.

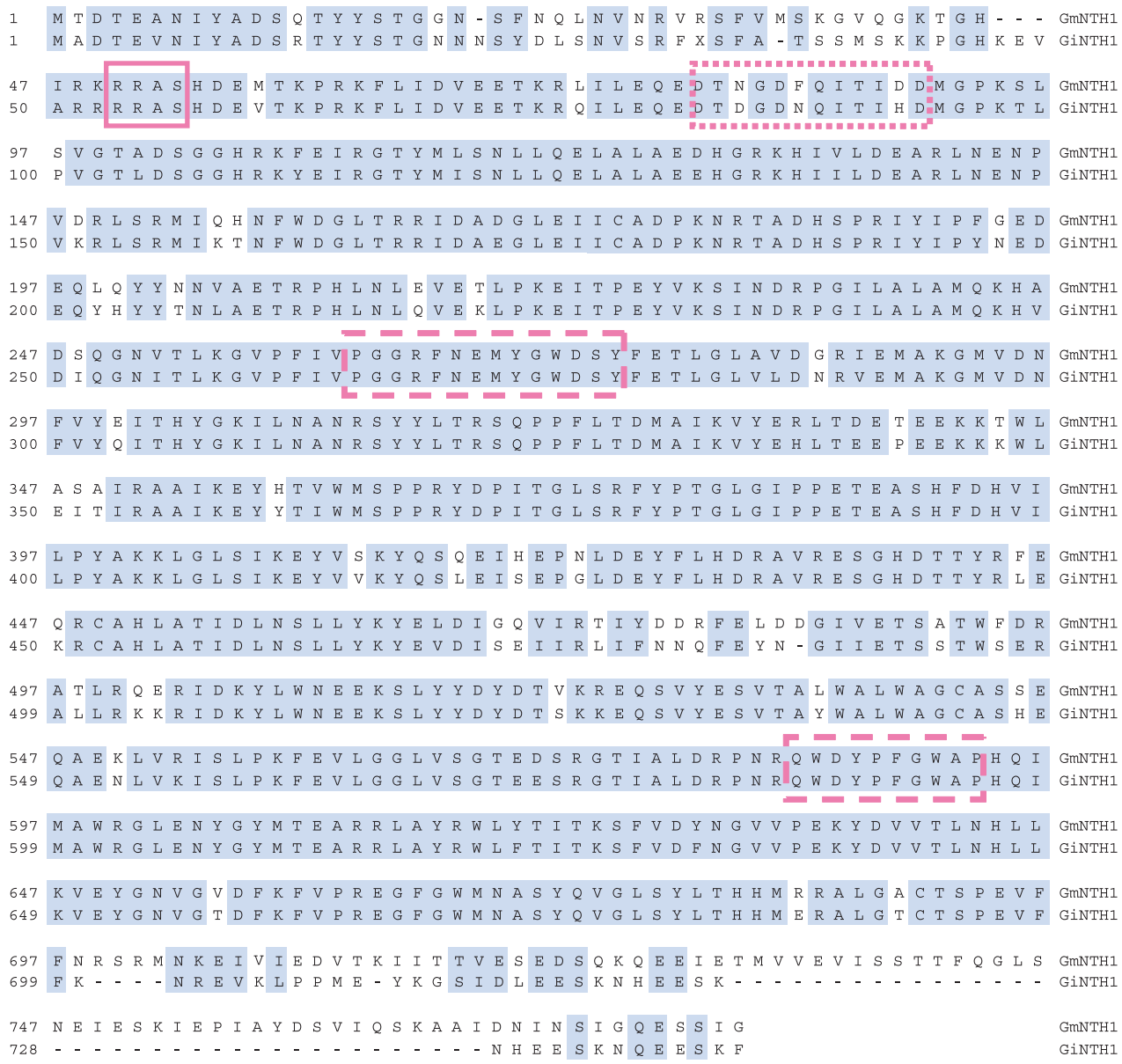

Fig. 2 Amino acid alignment of the *Glomus intraradices* and *G. mosseae* NTH1 deduced proteins. Amino acids in the solid box indicate a putative phosphorylation site by cAMP-dependent protein kinase (RRAS). Amino acids in the dashed boxes correspond to trehalase signatures 1 and 2. A putative EF-like Ca^{2+}-binding site is in the dotted box.

The genomic sequence encoding the ORF of *GmNTH1* was isolated by PCR using genomic DNA of *G. mosseae* as a template. Sequencing of the genomic fragment (3367 bp) showed the presence of three introns, located at positions +105, +163 and +1665 from the ATG. The introns had a size between 77 and 335 bp (data not shown).

Alignment of *G. mosseae* and *G. intraradices* NTH1 with 21 other neutral trehalases showed that the AM fungal proteins are highly conserved. A radial phylogenetic tree generated from the multiple alignment using the neighbor-joining algorithm implemented on the computer program CLUSTAL X was constructed. Several clusters were identified corresponding to filamentous ascomycetes, yeast ascomycetes, basidiomycetes and higher eukaryotes (Fig. 3a). Interestingly, and similar to what we described for two AM fungal glutamine synthetase genes (Breuninger *et al.*, 2004), *G. mosseae* and *G. intraradices* neutral trehalases emerge from the same branch originating from the basidiomycete cluster.

Heterologous complementation of a neutral trehalase yeast mutant with *GmNTH1*

The neutral trehalase-yeast mutant T19dN-kanMX4 was transformed with pAO196GmNTH1, expressing *G. mosseae*

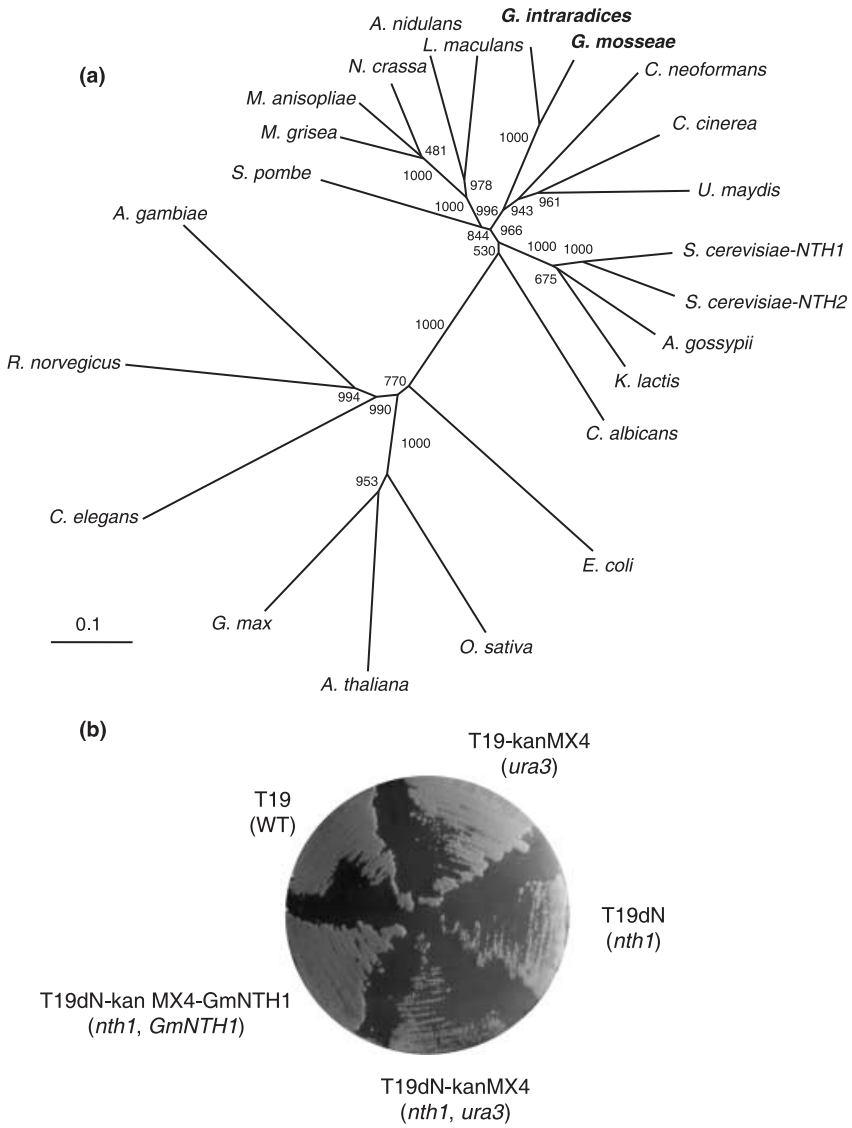

Fig. 3 (a) Phylogenetic relationships among known neutral trehalases from different organisms. A radial phylogenetic tree was constructed with the neighbor-joining algorithm implemented on the computer program CLUSTAL X and represented with TREEVIEW. Bootstrap analysis was performed on 1000 random samples. Branches are drawn to scale (bar, 0.1 changes per site). The sequences used in this analysis were: fungi (*Glomus intraradices* and *Glomus mosseae NTH1*, *Schizosaccharomyces pombe* CAA11904, *Saccharomyces cerevisiae NTH1* P32356, *S. cerevisiae NTH2* P35172, *Candida albicans* P52494, *Metarhizium anisopliae* AAT68667, *Magnaporthe grisea* O42622, *Neurospora crassa* O42783, *Aspergillus nidulans* O42777, *Ashbya gossypii* Q757L1, *Kluveromyces lactis* P49381, *Leptosphaeria maculans* AAM92145, *Ustilago maydis* EAK82334, *Cryptococcus neoformans* EAL22410, *Coprinopsis cinerea* EAU85396); plants (*Arabidopsis thaliana* AAF22127, *Glycine max* AAD22970, *Oryza sativa* AAG13442); metazoa (*Caenorhabditis elegans* AAF98588, *Rattus norvegicus* XP_343381, *Anopheles gambiae* EAA00681); and bacteria (*Escherichia coli* E90841). (b) Complementation with the full-length cDNA of *GmNTH1* (T19dN-kanMX4-GmNTH1) restores the wild-type phenotype of the neutral trehalase mutant strain (T19dN-kan MX4) that otherwise presents a poor heat shock recovery phenotype. Relevant genotypes are indicated in parentheses below the strain names.

neutral trehalase under the control of the constitutive yeast H^+-ATPase promoter (PMA1). Transformation efficiency was very high with an average of 10^6 transformants per 1.0 μg plasmid/10^8 cells per plate of selective medium. Selection was made on YNB without uracil for 2 d at 30°C. Ten colonies were PCR-analyzed to verify the presence of the *G. mosseae* gene. The analysis showed that nine out of 10 assayed colonies contained the *GmNTH1* cDNA (data not shown). Wild-type strains, neutral trehalase mutants, and transformants containing *GmNTH1* were subjected to a heat shock recovery assay. Results showed that strains expressing *GmNTH1* were able to recover after heat shock treatment, similar to the wild-type strains, and in contrast to the neutral trehalase mutant strains (Fig. 3b).

Changes in trehalose metabolism in response to stress

Because trehalose metabolism has been reported to play a role in the response to stress in several fungi, we monitored how extraradical hyphae from *G. intraradices* responded to several stress situations (heat shock, osmotic and chemical stress) with changes in trehalose turnover. Our results indicate that trehalose metabolism plays an important role in the response to heat stress and possibly in the response to arsenate stress.

When subjected to sublethal heat stress (10°C above normal growth temperature), trehalose concentration in hyphae steadily increased during the time-course of growth. After 5 h of exposure to 37°C, trehalose reached values 10-fold higher than in control hyphae grown at 27°C (Fig. 4a). Trehalose accumulation run parallel to a general increase in the activity of trehalose-6-P phosphatase and neutral trehalase for up to 2 h, after which time both activities decreased (Fig. 4b). No significant transcriptional activation of *GiNTH1* was observed upon heat shock (Fig. 4c).

A moderate transient increase was observed in *GiTPS2* RNA accumulation after 1 h of heat shock, parallel to a higher trehalose-6-P phosphatase activity with a maximum at 2 h.

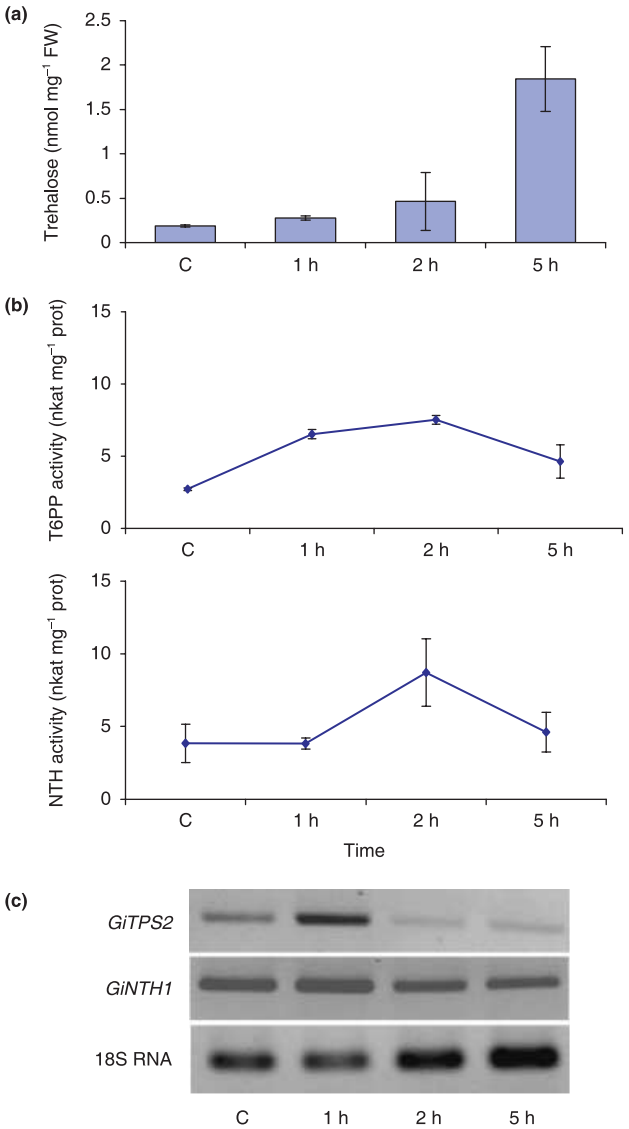

Fig. 4 Time-course of heat shock effects on trehalose metabolism in *Glomus intraradices* extraradical hyphae. Trehalose content (a), activities of trehalose 6-phosphate phosphatase (T6PP) and neutral trehalase (NTH) (b), as well as expression of *GiTPS2* and of *GiNTH1* (c). Data were obtained from control samples grown at 27°C for 5 h (C) and from samples subjected to heat shock (37°C) for 1, 2 and 5 h. RNA was calibrated using the 18S ribosomal RNA as control.

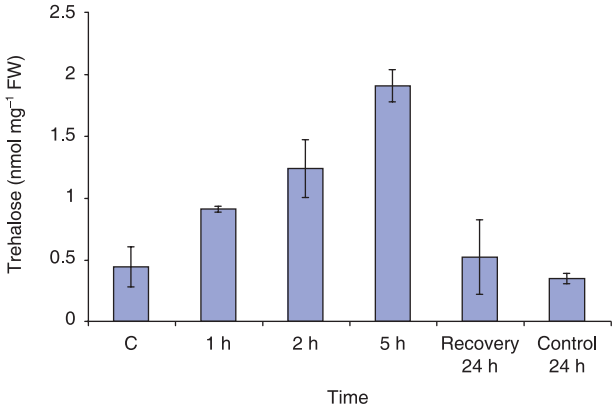

Fig. 5 Trehalose content during heat shock and recovery in *Glomus intraradices* hyphae. Data were obtained from control samples grown at 27°C for 5 h (C) and from samples subjected to heat shock (37°C) for 1, 2 and 5 h. Recovery samples, control and stressed, were allowed to grow at 27°C for a further 24 h and then harvested.

This was accompanied by an increased rate of activity of neutral trehalase at that time point. However, after 5 h of exposure to heat stress both enzymes exhibit reduced activities, compared with the 2 h values.

In support of a role in trehalose mobilization after heat stress of AM neutral trehalases, we successfully complemented the yeast mutant defective in neutral trehalase using the gene *GmNTH1* (Fig. 3b). Following transformation and heterologous expression of *GmNTH1* under the control of the PMA1 constitutive promoter, the mutant strain was able to recover after heat shock similar to the wild-type strain. Because this suggests that neutral trehalase could also be involved in the recovery after heat shock by activating trehalose hydrolysis, we performed a recovery experiment. Hyphae were allowed to recover for 24 h at 27°C (normal growth temperature) after 5 h of heat shock (37°C). Trehalose measurements showed that concentrations of trehalose after the 24 h recovery period were back to basal values (Fig. 5).

Osmotic stress did not appear to induce major changes in trehalose metabolism in extraradical hyphae of *G. intraradices*. Trehalose content was not altered in response to 0.5 M NaCl (Fig. 5a). Only moderate transient activations of TPS2 and NTH1 activities not associated with any transcriptional change were observed (Fig. 6b,c).

By contrast, chemical stress caused by arsenate induced changes in neutral trehalase activity which increased steadily with time. This was not accompanied by changes in GiNTH1 transcript level. By contrast, expression analysis of trehalose-6-P phosphatase showed an up-regulation in response to arsenate after 40 min, which correlated with both an increase in trehalose-6-P phosphatase activity and an accumulation of trehalose in the cell (Fig. 7a–c).

Discussion

The best-studied trehalose-inducing condition in fungi is heat stress. In response to mild and severe heat shock, fungal cells respond by activating transcriptionally and/or post-transcriptionally enzymes of the trehalose metabolism with an overall effect of trehalose accumulation (de Virgilio *et al.*, 1991; Cansado *et al.*, 1998a,b; Fillinger *et al.*, 2001; Van Dijck *et al.*, 2002; Gancedo & Flores, 2004). Interestingly, not only enzymes from the trehalose synthase complex are induced upon heat and other stresses but also neutral trehalase, responsible for the internal trehalose breakdown. This phenomenon of

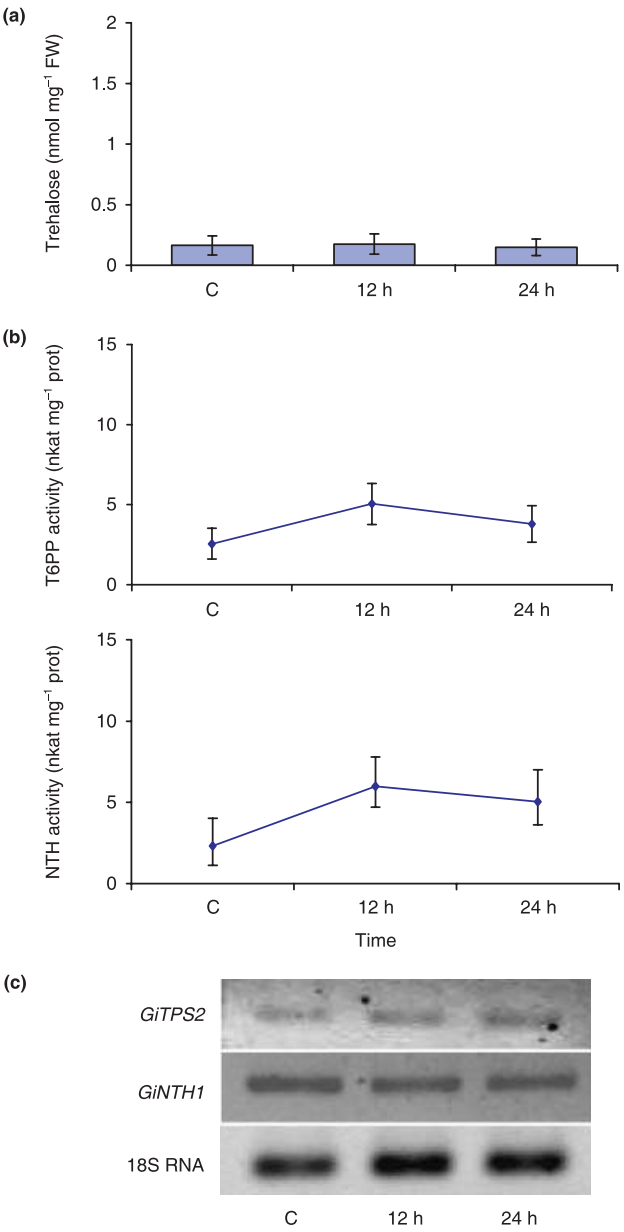

Fig. 6 Time-course of osmotic stress effects on trehalose metabolism in *Glomus intraradices* extraradical hyphae. Trehalose content (a), activities of trehalose 6-phosphate phosphatase (T6PP) and neutral trehalase (NTH) (b), as well as expression of *GiTPS2* and of *GiNTH1* (c). Data were obtained from control samples grown at 27°C for 24 h (C) and from samples subjected to osmotic stress (0.5 M NaCl) for 12 and 24 h. RNA was calibrated using the 18S ribosomal RNA as control.

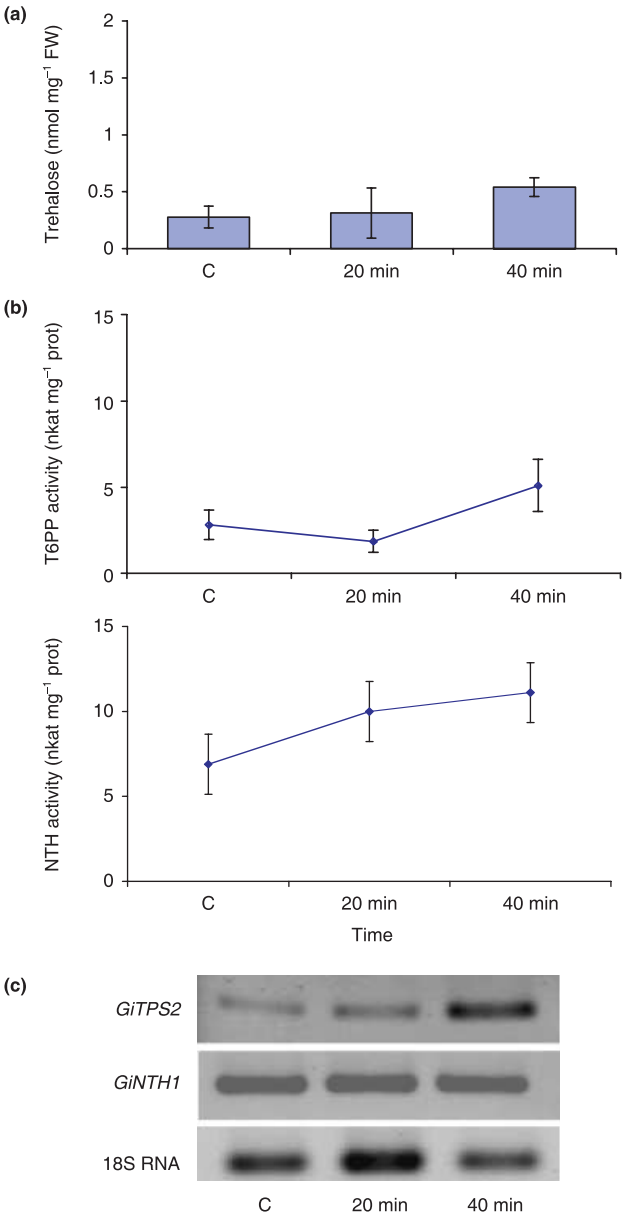

Fig. 7 Time-course of chemical stress effects on trehalose metabolism in *Glomus intraradices* extraradical hyphae. Trehalose content (a), activities of trehalose 6-phosphate phosphatase (T6PP) and neutral trehalase (NTH) (b), as well as expression of *GiTPS2* and of *GiNTH1* (c). Data were obtained from control samples grown at 27°C for 40 min (C), and from samples subjected to chemical stress (1 mM As_2HO_4) for 20 and 40 min. RNA was calibrated using the 18S ribosomal RNA as control.

trehalose futile cycling with activation of anabolic and catabolic enzymes during heat stress was first observed in the yeast *S. cerevisiae* (Hottiger *et al.*, 1987). In order to gain an insight into how trehalose metabolism was affected by heat stress and other stresses in AM fungi, we assessed trehalose content as well as enzyme activity of neutral trehalase and trehalose-6-P phosphatase in *G. intraradices*. To complement this data we also isolated the full-length cDNA of a neutral trehalase and partial cDNA of a trehalose-6-P phosphatase and studied their RNA accumulation during abiotic stress. Interestingly, we found that sublethal heat shock consisting of an increase of 10°C above the normal growth temperature induces trehalose accumulation in *G. intraradices* and this is possibly the result of a combined action of both synthesis and

degradation activities. Neutral trehalase appears to be an important enzyme for the recovery from heat shock in AM fungi. Increase of neutral trehalase activity during heat shock has been extensively studied in *S. cerevisiae* and *S. pombe* and found to be mediated by both transcriptional and post-translational regulation by cAMP-dependent protein kinase A (PKA)-mediated phosphorylation (de Virgilio *et al.*, 1991; Nwaka *et al.*, 1995; Ribeiro *et al.*, 1997; Cansado *et al.*, 1998a; Zähringer *et al.*, 1998). In contrast to yeast, we did not observe significant transcriptional activation of GiNTH1 upon heat shock, despite the increase of activity at 2 h, suggesting post-translational regulation of the AM fungal enzyme as the main activation mechanism under these conditions. However, we cannot exclude the possibility that other neutral trehalase genes exist and are regulated in a different manner. Similarly to GiNTH1, neutral trehalases from the filamentous fungi *A. nidulans* and *N. crassa* were not transcriptionally activated by heat shock (d'Enfert *et al.*, 1999), although recovery from heat shock in *A. nidulans* produced a modest but significant RNA accumulation of the corresponding genes that, together with a post-translational activation, could explain the trehalose mobilization observed during heat shock recovery in these fungi (d'Enfert *et al.*, 1999). The presence of a putative cAMP-dependent protein kinase phosphorylation site in the neutral trehalase deduced protein in both AM fungi studied is consistent with a post-transcriptional regulation via cAMP-dependent protein kinase (PKA) by phosphorylation, as has been shown for other fungi (Amaral *et al.*, 1997). It is also in agreement with our results, where maximum neutral trehalase activity was achieved after addition of cAMP to the reaction mix (data not shown). The EF-like Ca^{2+}-binding site localized at the N-terminus of both AM fungal proteins has also been identified in all fungal neutral trehalases. This motif could contribute to a further regulation of enzyme activity, as has been proposed for other fungi (Amaral *et al.*, 1997; Franco *et al.*, 2003).

Heat shock also induces expression of TPS2 and its ortholog tpp1+, coding for the phosphatase of the trehalose synthase complex, in *S. cerevisiae* and *S. pombe*, respectively. This runs parallel with an increase in trehalose-6-P phosphatase activity (de Virgilio *et al.*, 1993; Franco *et al.*, 2000). Similarly, we observed a moderate transient increase in GiTPS2 RNA accumulation after 1 h of heat shock, parallel to a higher trehalose-6-P phosphatase activity with a maximum at 2 h. This, together with an increased rate of activity of neutral trehalase indicates an increased turnover of trehalose, because its pool size is only slightly elevated (Fig. 4a). However, after 5 h of exposure to heat stress, both enzymes exhibit reduced activities, compared with the 2 h values. Trehalose concentrations after 5 h of heat stress undergo a 10-fold increase with respect to the control values under normal growth conditions. This increase is of a similar magnitude to that observed in *C. albicans*, where heat shock produces a trehalose increment from 5 to 50 nmol trehalose mg^{-1} fresh weight (Zaragoza *et al.*, 1998). We thus assume that in the period between 2 and 5 h of stress treatment, down-regulation of the *G. intraradices* neutral trehalase precedes that of the trehalose phosphatase, in order to achieve the required amount of trehalose able to protect from the stress. This could be achieved by a faster degradation of the neutral trehalase enzyme.

In yeast and other fungi, disruption of the trehalose-6-P phosphatase gene leads, in parallel to the abolition of trehalose-6-P phosphatase activity, to a thermosensitive phenotype of different magnitudes (Piper & Lockheart, 1988; de Virgilio *et al.*, 1993; Borgia *et al.*, 1996; Franco *et al.*, 2000; Van Dijck *et al.*, 2002; Zaragoza *et al.*, 2002). Interestingly, in *S. cerevisiae* the thermosensitive phenotype of TPS2 mutants was found to be the result of an accumulation of the intermediate trehalose-6-phosphate rather than a decrease in trehalose concentration (Elliot *et al.*, 1996). Experiments in *A. nidulans* and *C. albicans* using trehalose-6-P phosphatase mutants indicate a possible correlation between the accumulation of trehalose-6-phosphate and defects in the cell wall biosynthesis/assembly (Borgia *et al.*, 1996; Zaragoza *et al.*, 2002). How do increases in neutral trehalase activity observed during the time-course of heat shock in *G. intraradices* fit into this model? A possible hypothesis is a two step-protection mechanism vs heat shock, where the cell would first react to maintain low trehalose-6-phosphate concentrations. A decrease in the amount of trehalose-6-phosphate would be achieved by pushing the reaction to trehalose formation (increased GiTPS2 activity) and further to glucose (increased GiNTH1 activity). Both activities would have to be coordinated to produce a net increase of trehalose that would serve as a protectant for, for example, soluble proteins or cell structures. In a second step, net trehalase activity would have to prevail in order to allow trehalose mobilization for recovery after heat shock. Similar distinct roles for the neutral trehalase activity during heat shock and heat shock recovery have been postulated for *A. nidulans*, *N. crassa* and *S. cerevisiae* (d'Enfert *et al.*, 1999; Wera *et al.*, 1999).

In support of a role in trehalose mobilization after heat stress of AM neutral trehalases is the successful complementation of the yeast mutant defective in neutral trehalase with GmNTH1 (see earlier discussion). This mutant, similar to other neutral trehalase yeast mutants, displays the so-called 'poor heat shock recovery phenotype' (Nwaka *et al.*, 1995). This is because the lack of neutral trehalase activity prevents the strain from growing properly after 6 h at 50°C. Following transformation and heterologous expression of GmNTH1 under the control of the PMA1 constitutive promoter, the mutant strain was able to recover after heat shock similar to the wild-type strain. It is interesting that despite the fact that GmNTH1 presents higher similarity to the basidiomycetes than to yeast neutral trehalases, the AM fungal protein is able to complement the mutant phenotype. This is not surprising considering that yeast has been proven to be a good model organism for heterologous complementation even across

kingdoms (Piotrowski *et al.*, 1998), and has been successfully used for several AM fungal genes (Harrison & van Buuren, 1995; Lanfranco *et al.*, 2005). The functional complementation analysis in yeast using the neutral trehalase gene of *G. mosseae* confirms the role of neutral trehalase in the recovery after heat shock and proves the functionality of an AM neutral trehalase in this process. Additionally, it suggests that neutral trehalase could promote the recovery after heat shock by activating trehalose hydrolysis. This was confirmed in the recovery experiment, in which concentrations of trehalose quickly returned to basal values after the 24 h recovery period, indicating an active role of the AM neutral trehalase in mobilizing accumulated trehalose after the stress period.

In contrast to the well-established protective role of trehalose during heat stress, it appears that only extreme forms of osmotic stress, such as desiccation and freezing, clearly involve trehalose as an osmoprotectant in yeast (reviewed in Hohmann, 2002). However, in different fungi, moderate osmotic stress induces different trehalose phenotypes. Thus, in *S. pombe*, there was increased neutral trehalase expression and activity as well as trehalose-6-P phosphatase activity concomitant with increased concentrations of trehalose after exposure to 0.75 M NaCl (Cansado *et al.*, 1998a; Franco *et al.*, 2000). *Candida albicans* is in general much less effective than *S. cerevisiae* in accumulating compatible solutes during stress. However, in contrast to *S. cerevisiae,* osmotic stress (0.3 M NaCl) induces trehalose accumulation and transcription of trehalose-related enzymes to a greater degree than heat or oxidative stress (Enjalbert *et al.*, 2003). In *S. cerevisiae* 0.5 M NaCl does not induce significant changes in the trehalose content of cells despite increased enzyme activities of TPS1 and NTH1 (Zähringer *et al.*, 2000). The phytopathogenic fungus *Botrytis cinerea*, which undergoes activation of trehalose metabolism in response to heat stress, is also not responsive to osmotic stress induced by NaCl (0.5–1.5 M) or by sorbitol (0.5–1.0 M) (Döhlemann *et al.*, 2006). Similarly to these two latter fungi, we observed that *G. intraradices* did not alter its trehalose content in response to 0.5 M NaCl and only underwent moderate transient activations of TPS2 and NTH1 not associated to any transcriptional change.

It has been suggested that chemical stressors (such as arsenite) may modify protein activity or structure by reacting with thiol groups and thus transiently affect cell growth (Chang *et al.*, 1989). Since toxic chemicals did not lead to an increased trehalose concentration in *S. cerevisiae* cells, it was suggested that an increase in neutral trehalase activity could indicate a direct participation of the enzyme in the defense or detoxification mechanism against this chemical (Zähringer *et al.*, 1997). However, in our study we observed both an increase in neutral trehalase activity and a small but significant increase in trehalose content in response to arsenate. These results could suggest that, in contrast to *S. cerevisiae*, not only the neutral trehalase enzyme, but also trehalose, is involved in the protection of the fungus against arsenate. It has been shown that mycorrhizal plants accumulate less arsenate than nonmycorrhizal plants when growing on contaminated soils (Liu *et al.*, 2004). As arsenate is transported by the same transporters as orthophosphate, the increased phosphate amount in mycorrhizal plants could account for a lower arsenate accumulation ratio. Since trehalose formation yields inorganic phosphate, this metabolic step has been suggested as a control point for orthophosphate liberation with possible implications in the glycolysis control in yeast (Thevelein & Hohmann, 1995). The trehalose synthase complex is activated allosterically by fructose-6-phosphate and inhibited by free phosphate, implying it would have maximum activity when sugar phosphates accumulate and phosphate drops. NMR studies of mycorrhizal roots show that import of hexoses by intraradical hyphae is quickly channelled to trehalose formation (Shachar-Hill *et al.*, 1995; Pfeffer *et al.*, 1999). In addition, intraradical hyphae are responsible for the transfer of phosphate to the root. In this scenario, one could speculate that synthesis of trehalose/liberation of inorganic phosphate within the fungus stimulated by the hexose import from the plant could favor phosphate liberation/translocation to the plant. Under arsenate stress, increases in trehalose synthesis would fuel this phosphate translocation and decrease the relative accumulation of the toxic arsenate in the plant. At high enough rates of trehalose turnover and sugar phosphate/trehalose shuttling, even the observed low concentrations of trehalose would be sufficient.

Conclusions

We present evidence here that in symbiotic AM fungi, similar to other organisms, trehalose and trehalose metabolism could play an important role in the protection of the cell from several abiotic stresses. In particular, trehalose accumulation and mobilization in response to heat shock and arsenate stress might be of ecological relevance. Fungi as well as plants are sessile organisms that are highly exposed to disturbances in the environment. Their ability to react and protect themselves depends almost exclusively on cellular mechanisms to tolerate stress situations. More studies are needed to evaluate the potential of mycorrhizal colonization and the molecular mechanisms involved in the protection of plants under stress conditions.

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