Journal of Cell and Molecular Research (2010) 2 (1), 1-9

Characterization of *Arabidopsis* seedlings growth and development under trehalose feeding

Mahnaz Aghdasi[1,2]*, Henriette Schluepmann[2] and Sjef Smeekens[2]

[1]*Department of Biology, Faculty of Science, Golestan University, Iran*
[2]*Department of Molecular Plant Physiology, Institute of Environmental Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*

Received 20 June 2010 Accepted 6 September 2010

Abstract

Trehalose is the alpha, alpha-1, 1-linked glucose disaccharide. Its metabolism is found in a wide variety of organisms and is seen as evolutionary old. Trehalose metabolites are, however, present at only very low concentrations and their role in plants are not understood. The physiological effects of 100 mM trehalose on growth and carbon allocation in seedlings are characterized in this paper. Trehalose feeding to *Arabidopsis thaliana* elicits strong responses. On 100 mM trehalose, seedlings germinate and extend cotyledons but fail to develop primary leaves. The primary roots do not grow beyond 2-3 mm and there is not any starch in root tips. In light, growth arrest on 100 mM trehalose can be rescued by exogenous supply of metabolisable sugar. Trehalose feeding results in anthocyanin accumulation and chlorophyll reduction. Trehalose causes cells of the root extension zone to swell and lysis. Trehalase expression analysis showed that WT seedlings grown on trehalose have 10-fold induced *AtTRE1* expression compared to the sorbitol treatment.

***Keywords*:** trehalose, T6P, trehalase, carbon allocation, growth, Arabidopsis

Introduction

Trehalose is the alpha, alpha-1, 1-linked glucose disaccharide, which is found ubiquitously and is therefore thought to be evolutionary ancient (Elbein et al., 2003). Its metabolism has recently been recognized to play an important role in carbon signaling in plants (Paul et al., 2008; Rolland et al., 2006). Trehalose is a carbon reserve. It has been shown that Trehalose has several biological functions. In fungal spores, trehalose hydrolysis occurs during early germination and presumably provides glucose for energy and biosynthesis (Thevelein, 1984). Trehalose is a stress protectant and protects proteins and membranes from denaturation by replacing water as it makes hydrogen bonds to polar residues (Brumfiel, 2004; Croweet et al., 1998; Wolkers et al., 2003). Plants generally contain only trace amounts of trehalose (Muller et al., 1995; Zentella et al., 1999). Exceptions to this exist and these are plants with extreme drought stress resistance such as *Selaginella lepidophylla* that accumulate quantitative amounts of trehalose. All plants seem to contain genes for trehalose metabolism (Blazquez et al., 1998; Leyman et al., 2001; Shima et al., 2007; Vogel et al., 1998). Synthesis of trehalose in plants is typically via its phosphorylated intermediate, trehalose-6-phosphate (T6P). Trehalose-6-phosphate synthase (TPS) converts UDP-Glucose and Glucose-6-phosphate to T6P. Trehalose phosphate phosphatase (TPP) de-phosphorylates T6P to trehalose. Trehalase cleaves trehalose to two glucose molecules (Elbein et al., 2003). Moreover, it has been shown that *Arabidopsis* encodes a single active TPS and a family of putative TPS-like proteins that have specific regulatory functions in actively growing tissues (Vandesteene et al., 2010).

Minor alterations of T6P steady states in plants yield dramatic and pleiotropic phenotypic changes (Pellny et al., 2004; Pramanik and Imai, 2005; Schluepmann et al., 2003; Schluepmann and Paul, 2009). Additionally, deletion of the T6P synthase (TPS), gene *AtTPS1*, in Arabidopsis is lethal and can be overcome by complementation with active TPS enzyme (Eastmond et al., 2002; Schluepmann et al., 2003). Evidences are thus accumulating that suggest an important regulatory role for T6P in the coordinating of metabolism with development (Paul et al., 2008). It is not understood, however, how

∗Corresponding author E-mail:
aghdasi@gu.ac.ir

T6P controls carbon utilization and why changes in the steady state level of T6P yield such strong phenotypic changes.

Attempts to produce trehalose in plants by over-expressing yeast *TPS* in tobacco yielded drought resistant plants (Holmstrom et al., 1996; Romero et al., 1997). Expression of *E.coli TPS-TPP* fusions in rice also yielded drought tolerance and in addition salt tolerance (Garg et al., 2002; Jang et al., 2003). Trehalose metabolism has been implicated in biotic stress resistance as well. Spraying wheat with a trehalose solution confers resistance to *Blumeria graminis* infection. Trehalose appears to activate plant defense responses e.g. papilla deposition, phenylalanine ammonia lyase and peroxidase activities (Reignault et al., 2001). The data suggests that trehalose and/or T6P may be a key component in plant-microorganism interactions (Iturriaga et al., 2009). The underlying mechanism is unclear so far. Isolation and characterization of *Arabidopsis* mutants resistant to exogenous trehalose at 100 mM could be a main achievement in understanding trehalose mechanisms against biotic and abiotic stresses.

In the present work, the Arabidopsis seedlings of growth inhibition due to T6P accumulation on ½ MS medium supplemented with 100 mM trehalose is characterized further. This characterization is necessary since the effects of 100 mM trehalose may be different from the effects of 25 mM trehalose combined with 10 µM Validamycine A that were used previously to describe the effect of trehalose (Fritzius et al., 2002; Roman et al., 2007; Wingler et al., 2000). The characterization of the physiological effects of 100 mM trehalose on seedlings presented in this paper will enable us to isolate and interpret the mutants from the suppressor screen. Results showed that seedlings that had long roots with primary and secondary leaves, high level of T6P and low level of starch after growing on 100 mM trehalos could be used as trehalose resistant mutants.

Material and Methods

Plant material and growth conditions

In this study, *Arabidopsis thaliana* accession Columbia-0 was used as Wild type (WT). Seeds of WT and TreF 46.2, a line expressing *E.coli* cytosolic trehalase behind the CaMV35S promoter (Schluepmann et al., 2003) were used in this study. Seeds were sterilized 5 minutes with 70% Ethanol followed by 10 minutes in 20 % commercial bleach (4% w/v chlorine) and washed 5 times in sterile milli-Q water. Sterilized seeds were plated on agar solidified half strength MS medium (Murashige and Skoog, 1962) supplemented with 50 mM trehalose with or without 50 mM sugars (sucrose, maltose, glucose, fructose, sorbitol and palatinose) and stratified in darkness at 4ºC for 2 days before the plates were transferred to a growth chamber at 25ºC under a 16-h-light/8-h-dark photoperiod. In this experiment, seedlings were grown vertically for 14 days. After 7 days, pictures were taken and the root length was measured with the Image J program (Wayne Rasband, NIH Maryland, USA).

Starch staining and confocal microscopy

For analysis of starch distribution, all the seedlings were taken and destained in 70% and then 90% v/v ethanol. Staining was done with Lugol solutions and then washed with milli Q water. Pictures were taken using a Normarski microscope (Jena, Germany). For confocal Laser scanning microscopy (Zeiss, Germany), the roots were stained with propidium iodine (1µg/ml).

Chlorophyll and anthocyanin measurements

Chlorophyll a, b and total chlorophyll were measured spectrophotometrically as described by Jeffery and Humphery (1975). In brief, 14 days seedlings were ground in liquid nitrogen and extracted with 80% v/v acetone. Then, the absorbance was measured (at 647, 652 and 664nm) and used to calculate chlorophyll content.

Anthocyanin content of seedlings was determined using the protocol of Mita et al., (1967). Frozen and homogenized seedlings (20 mg) were extracted for 1 day at 4°C in 1 mL of 1% (v/v) hydrochloric acid in methanol. The mixture was centrifuged at 13,000 rpm for 15 min and the absorbance of the supernatant was measured at 530 and 657 nm. Relative anthocyanin concentrations were calculated with the formula [A_{530} – (1/4 x A_{657})]. The relative anthocyanin amount was defined as the product of relative anthocyanin concentration and extraction solution volume. One anthocyanin unit equals one absorbance unit [A_{530} – (1/4 x A_{657})] in 1 mL of extraction solution.

T6P measurements

Fifty mg fresh weight of 14 days old seedling were snap frozen, then ground using a dismembranator (Braun, Germany) before extraction with 800 µl of chloroform/ acetonitrile/water at a ratio of 5:7:2 for 2 hours at –10°C. After 5 min 6000 g centrifugation (Braun, Germany) at 4°C for 5 min, the acetonitrile/water phase was recovered and the chlorophorm phase

back-extracted with 400 µl water, the water and acetonitrile water phases were combined and dried under vacuum over night. Samples were taken up in 1 ml water prior to solid phase extraction (SPE). After loading, the SPE phase was rinsed with 4 volumes 5 mM NaOH. Then, samples were eluted twice with 0.5 ml 2% v/v formic acid. Eluates were combined and dried under a flow of nitrogen, resuspended in 0.2 ml water, filtered and 10 µl injected onto the AS-11-HC column (250 ×2 mm, Dionex, USA) for HPLC-PAD (Dionex) or HPLC-MS (Ion trap, Agilent). The ion exchange column was eluted with a 5-100 mM gradient of NaOH. Addition of T6P (12.8 nmol) during the extraction and or immediately loading onto the HPLC allowed calculation of T6P recoveries and an approximate evaluation of amounts of T6P in the extracts.

RNA isolation, RT-PCR and quantitative PCR (Q-PCR) analysis

Seeds of *Arabidopsis thaliana* accession Columbia-0 (WT) were grown on ½ MS medium for 10 days. The plant material was snap frozen in liquid nitrogen, pulverized with glass beads for 2 minutes at 2800 rpm in a dismembrator (Braun, Germany). The total RNA was isolated with RNeasy plant mini kit (QIAGEN USA, Valencia, CA). The RNA concentration and purity were determined by measuring at 260 nm. 10 ng RNA was treated with 2 U DNAse I (DNA- free, Ambion, Austin, USA) to remove genomic DNA. Absence of DNA was analyzed by performing a PCR reaction (40 cycles, similar to the real–time PCR program) on the DNaseI- treated RNA using Taq-DNA polymerase. RT-PCR experiments were performed using 1 µg of total RNA extracted and used for first-strand cDNA synthesis with sixty units M-MLV Reverse Transcriptase (promega, Madison, WI), 0.5 µg of odT16v (custom oligo from invitrogen, Carlsbad, CA) and 0.5 µg random hexamer (invitrogen).

Q-PCR was carried out by ABI-prism 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA). Per each reaction, 12.5 µl of CYBR green PCR Master Mix (Applied Biosystems, UK) and 2.5 µl of trehalase specific primer (AtTRE1-F 5'-gctgcaccacgaaccagtaga-3' and AtTRE1-R 5'-ttcttcgttctccacgttgga-3'; Efficiency: 1.98) were used. Relative quantitation of gene expression was based on the comparative Ct method (User Bulletin No. 2: ABI PRISM 7700 Sequence Detection system, 1997) using *AtACTIN2* as a calibrator reference.

Results

100 mM trehalose cause an accumulation of starch in source and depletion in sink tissues

Supply of the 100 mM trehalose to the *Arabidopsis* seedlings (WT) led to the growth arrest and development arrest in leaves. In WT seedlings, the root length was very short (1.9± 0.6 mm after 14 days) and emergence of primary leaves was entirely inhibited. The trehalase expressing seedlings (TreF, line 46.2) had 12 times longer roots than WT ones after 14 days growth on 100 mM trehalose. TreF seedling root lengths on trehalose were as long as them on the sorbitol osmoticum control (figure 1).

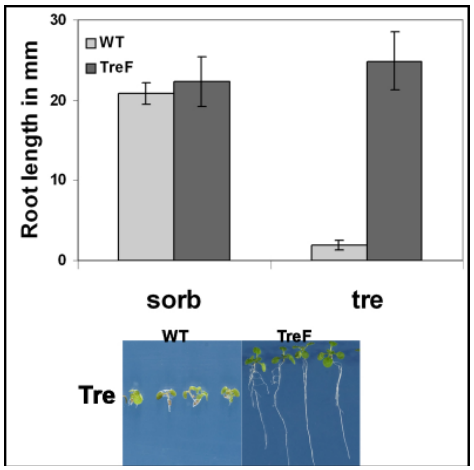

Figure 1. The effect of 100 mM trehalose on the root growth of WT seedlings. WT seeds were germinated and grown under long day conditions on ½ MS medium with 100 mM trehalose or sorbitol. Root length was measured after 14 days. Each experiment was repeated three times. Error bars indicate Standard deviation. The abbreviations are WT (Wild type), tre (trehalose), and sorb (sorbiyol).

Trehalose in the medium led to an accumulation of large amounts of starch in the seedling source tissue, cotyledon, and to a depletion of starch in the columnella cells of the root cap, a sink tissue (figure 2a-c). Confocal microscopy of the seedling roots stained with propidium iodine revealed swelling as well as lysis of the cells in the extension zone of roots grown on 100 mM trehalose but not on 100 mM sorbitol (figure 2d-f). In addition to altered starch distribution and reduced root growth, trehalose appeared to alter cell wall elasticity compared with sorbitol.

Distribution of starch in TreF and WT was studied in 14 d seedlings using Lugol staining. Staining revealed that the reaction to trehalose was not fully homogenous when examining a large number of WT seedlings: 72% of the seedlings responded with massive trehalose accumulation in the cotyledons whilst 28% failed to stain. The response to trehalose of seedlings expressing *E.coli* trehalase (TreF line) was homogenous, as cotyledons of these seedlings did not stain with Lugol. Seedlings of the TreF line displayed starch in the columnella cells of the root tips (not shown).

Quantification of starch in the WT and TreF seedlings on trehalose is shown in figure 2 g. WT seedlings contained 11 $mg \cdot g^{-1}$ FW (fresh weight) starch on medium with 100 mM sorbitol. On trehalose, the starch level in WT was increased to 52 $mg \cdot g^{-1}$ FW. TreF seedlings on trehalose contained the same amount of starch as WT on sorbitol.

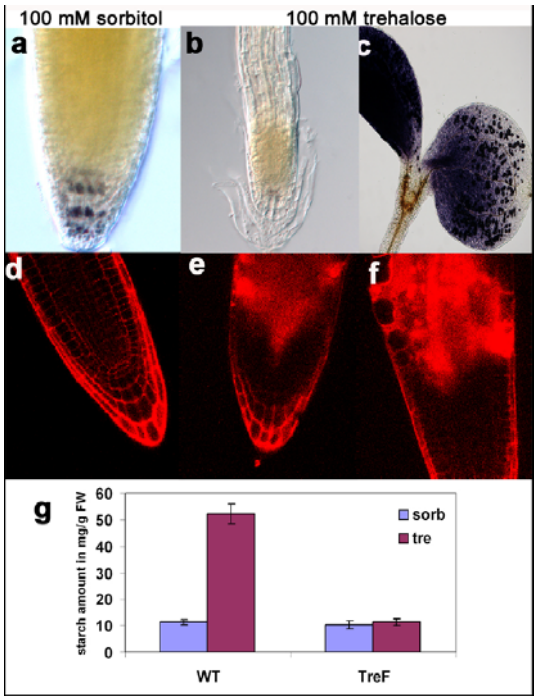

Figure 2. Starch staining and quantification. Seedlings were grown 14 d in long day conditions, then stained with KI/I_2 and studied using Nomarski microscopy. (a) Starch in the columnella of WT roots grown on 100 mM sorbitol osmoticum control, (b) Starch in the columnella of WT roots grown on 100 mM trehalose, (c) Starch accumulation in cotyledons of WT seedlings grown on 100 mM trehalose. Confocal microscopy of seedling roots with propidium iodine, (d) Typical root on 100 mM sorbitol osmoticum control, (e, f) typical swelling and lyses of cells at the extension zone of seedling roots on 100 mM trehalose, (g) Starch amounts of whole seedlings from WT and TreF on 100 mM sorbitol or trehalose after 14 days. The FW stands for Fresh Weight.

Trehalose-6-phosphate mediated growth arrest on trehalose is due to the altered carbon allocation

The seedling response to sugars without added trehalose was analyzed at 50 mM concentrations except for the toxic mannose where 6 mM was used (figure 3 without tre). The growth on 50 mM sorbitol, the osmoticum control equaled that on the half strength MS medium and thus suggests that osmoticum has little effect on growth at these sugar concentrations. Growth was enhanced when seedlings were supplied 50 mM of glucose, fructose, sucrose or maltose compared to the seedlings supplied with sorbitol; seedlings therefore utilized the supplied metabolisable carbon for growth. Interestingly, growth on the 100 mM of either fructose or glucose was not as vigorous as growth on 50 mM of each glucose and fructose or 50 mM of glucose or fructose. This was the case for both WT and trehalase expressing seedlings. Growth on palatinose equaled that on sorbitol

suggesting that this sugar, like sorbitol was not utilized. Trehalose at 50 mM inhibited root growth significantly to 30% of the control levels. Seedlings did not germinate on 50 mM 2-deoxy glucose or 6 mM mannose.

The seedling response to the 50 mM sugar combined with 50 mM trehalose was investigated (figure 3A, B). Sucrose, maltose and a combination of fructose and glucose completely alleviated the growth inhibitory effects of trehalose. Fifty milimolar of fructose or glucose alleviated the trehalose mediated growth inhibition partially. Sorbitol and palatinose were ineffective against the growth inhibition. The T6P accumulation on trehalose did not rescue inhibition of the seedling germination due to 2-deoxyglucose, and so T6P unlikely acted as an inhibitor of HXK2 mediated signaling. This was further supported by the fact that T6P accumulation did not rescue mannose inhibition of germination. After 7 days, growth of the TreF expressing seedlings on trehalose equaled that on sorbitol (figure 3A, C- with trehalose). If grown for longer periods of time, these seedlings thrived on trehalose with growth exceeding that on sorbitol, presumably because glucose from trehalose cleavage was used for the growth. Addition of sucrose, maltose or a combination of glucose and fructose increased growth of trehalase expressors on trehalose, their root lengths being twice as long on medium with trehalose and the metabolisable sugars.

Chlorophyll and anthocyanin contents correlate with the growth response

WT seedlings growing on trehalose had dark red-rimmed cotyledons. Chlorophyll and anthocyanin contents were determined in the WT and TreF seedlings. While total chlorophyll content of WT seedlings were reduced 4 fold after 14 days growth on trehalose compared to the growth on sorbitol

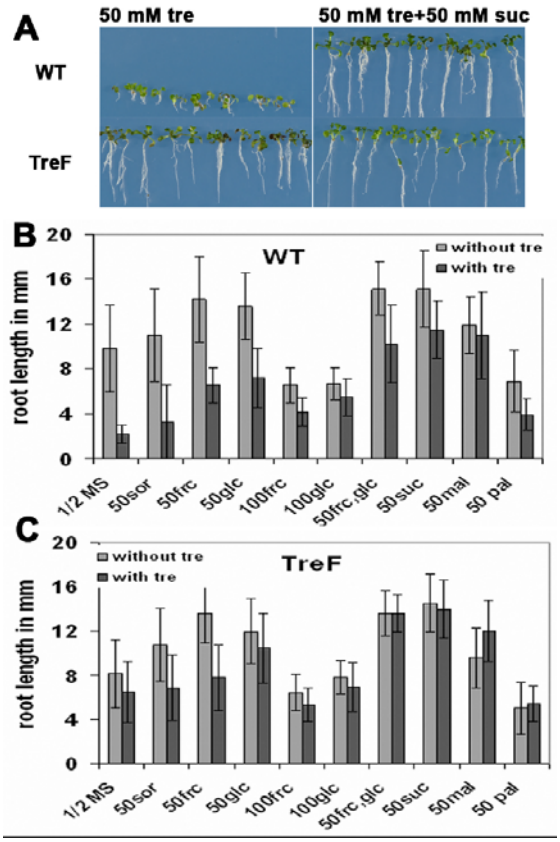

Figure 3. Effects of metabolisable sugar on the growth inhibitory effect of trehalose. Seedlings were grown for 14 days in long day conditions on half strength MS medium containing 50 mM trehalose with or without different sugars. Root lengths were determined using Image J. WT, seedlings from WT; TreF, seedlings from the *E.coli* trehalase, *TreF*, expressing line 46.2; (A) WT and TreF seedlings on 50 mM trehalose (50 mM tre) with or without 50 mM sucrose (50 mM tre+50 mM suc). (B) Root lengths of WT on half strength MS medium (1/2MS) with different sugar combinations. Sugars concentration in this experiment was at 50 mM, except for mannose, glucose, fructose and a combination of fructose and glucose, where the concentration were 6mM, 100 mM, 100 mM and 50 mM fructose combined with 50 mM glucose, respectively. (C) Root lengths of TreF in media with different combinations of sugar.

(figure 4a), the Chla/Chlb ratio increased by feeding of 100 mM trehalose (3.75 on trehalose compared to 2.13 on sorbitol).

Anthocyanin was 5 fold induced in the WT seedlings after 14 d growth on trehalose compared to sorbitol (figure 4b). The TreF seedlings did not show any change in chlorophyll and anthocyanin levels by the trehalose feeding (not shown).

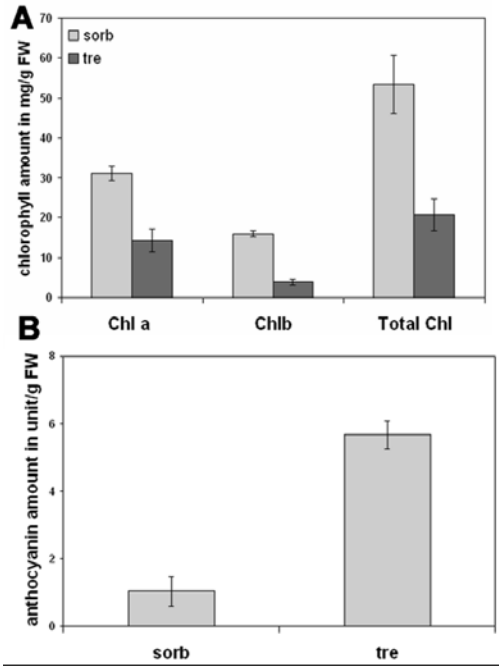

Figure 4. The effect of 100 mM trehalose on chlorophyll and anthocyanin content in WT seedlings. Seedlings were grown for 14 days on 100 mM of either sorbitol or trehalose in long day conditions. a) Chlorophyll and b) anthocyanin contents. Each experiment was repeated three times. Error bars indicate Standard deviation. WT: Wild Type, Chla: chlorophyll A, Chlb: chlorophyll B, Total Chl: total chlorophyll, FW: fresh weight.

T6P measurements

T6P levels were determined in 50 mg FW tissue using a pre-purification SPE procedure followed by HPLC-PAD and HPLC-MS. Results confirmed an accumulation of T6P in the WT seedlings grown for 14d on trehalose. The T6P level in 14d seedlings was 10.2 ± 2.5 nmol·g^{-1} FW, compared to the 2.78 ±1.3 nmol·g^{-1} FW on sorbitol (figure 5a).

Trehalase expression

We analyzed the expression levels of the *AtTRE1*gene, the only trehalase gene in Arabidopsis, to find out the trehalase expression status in WT seedlings growing on 100 mM trehalose. The analysis was performed using mRNA from 10 d old seedlings grown on ½ MS medium with 100 mM sorbitol or trehalose, and Q-PCR technique. Results showed that WT seedlings grown on trehalose had 10-fold induction of the *AtTRE1* expression compared to sorbitol (figure 5b).

Discussion

Trehalose supplied to the growth medium of seedlings inhibits growth and allocation of carbon to the root and shoot (Fritzius et al., 2001; Schluepmann et al., 2004; Schluepmann and Paul, 2009; Wingler et al., 2000). This growth inhibition was previously studied by combining 25 mM trehalose with 10 μM Validamycine, a trehalase inhibitor. In this research, we used 100 mM trehalose without addition of any trehalase inhibitor.

Supplied trehalose is transported through the plant tissues and enters the cells, since plants expressing trehalase in the cytosol thrive on medium with trehalose (Schluepmann et al., 2003; Schluepmann et al., 2004). In the light conditions, growth arrest on trehalose is due to T6P accumulation (Schluepmann et al., 2004; Schluepmann and Paul, 2009) and chlorophyll reduction in the WT seedlings implies that these typical senescence processes correlate with growth inhibition. T6P measurement carried out in this research revealed that trehalose is absorbed by the seedlings leading to an increase in the steady state of T6P supporting the notion that growth arrest of the seedlings on trehalose is due to T6P accumulation.

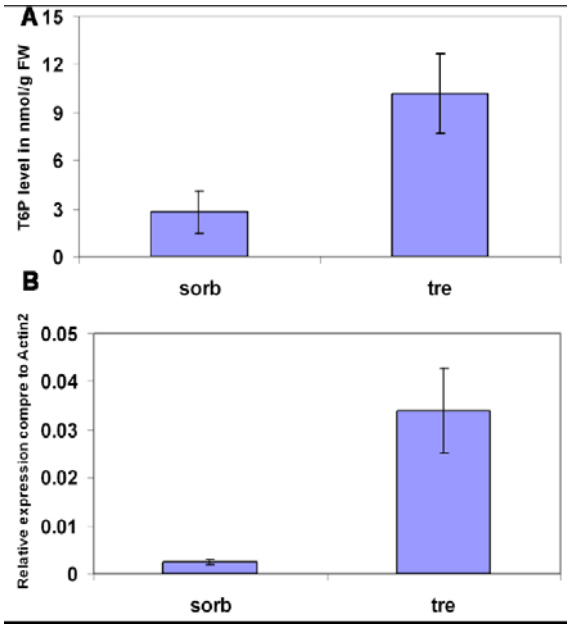

Figure 5. T6P quantification and trehalase gene expression. a) T6P level in WT. Seedlings of WT were grown for 14 days on half strength MS supplemented with 100 mM of either sorbitol (sorb) or trehalose (tre). T6P was quantified by HPLC-MS Scanning, b) Trehalase gene expression in WT. Seedlings were grown on agar solidified half strength MS for 10 days before RNA extraction and Q-PCR analysis of gene-expression. Levels of gene expression were determined with reference to *AtACTIN2*. Each experiment was repeated three times. Error bars indicate Standard deviation. WT stands for Wild Type.

The trehalose pathway regulates carbon partitioning in light

Accumulating evidences suggest an important regulatory role for T6P in growth and development of the seedlings (Paul et al., 2008; Schluepmann et al., 2003).

It is not understood however, how T6P controls carbon utilization and why changes in the steady state level of T6P yield such strong phenotypic changes.

Growth inhibition by trehalose can be overcome by expression of *E.coli* trehalase, TreF, a soluble enzyme targeted to the cytosol suggesting that trehalose supplied exogenously is imported into the plant cells, then cleaved by expressed trehalase and the released carbon is utilized for growth (Schluepmann et al., 2003). Growth inhibition on trehalose can be overcome by expression of the *E.coli* trehalose phosphate hydrolase, an enzyme that cleaves T6P into Glucose-6-phosphate and glucose suggesting that T6P accumulation is causing growth arrest (Schluepmann et al., 2004). Studying the effect of sugars on T6P mediated growth arrest may therefore reveal interactions between T6P and sugar signaling pathways that control carbon utilization in the source tissues or that control carbon allocation and transport.

The T6P-mediated inhibition of growth is likely due to starvation of the sink tissues important for growth, such as shoot and root apical meristems. It has been shown on 25 mM trehalose that carbon allocation is reversed by high accumulation of starch in cotyledons (Wingler et al., 2000). Interestingly, supply of the metabolisable sugar in addition to 100 mM trehalose relives the growth inhibitory effects of trehalose suggesting that starvation causes growth arrest; it further suggests that trehalose does not affect the ability of sink tissue to metabolise the allocated carbon. Increased elasticity of the cell walls in the extension zone and absence of starch accumulation in columnella cells of the root tip suggest that T6P accumulation throughout the plant tissues likely causes starvation of the sink tissues important for growth, such as shoot and root apical meristems. Sink starvation is not caused by the sink's inability to metabolize carbon since the supplied carbon is utilized and the effects of the T6P accumulation are then overcome. Indeed, starch is no longer formed at the root tip in the columnella when the WT seedlings are grown on 100 mM trehalose (Ramon et al., 2007; Wingler et al., 2000). Since *pgm1* (*pgm1* cannot synthesize starch) seedlings are also growth arrested on trehalose (Fritzius et al., 2001), T6P inhibition of the growth is not due to the carbon partitioning into starch in the cotyledons. Inhibition, therefore, is more likely due to carbon loading/unloading or transport. These effects can be at subcellular level, for example, export from chloroplast to the cytosol. Alternatively, that can be at the plant level i.e. the

interface between mesophyll and vascular bundles or simply involve transport inhibition in the phloem.

This can be exploited in a genetic screen program to identify plants altered in T6P metabolism or target processes of T6P. Mutant seedlings that overcome growth arrest on 100 mM trehalose are either altered in trehalose import, trehalose catabolism, T6P synthesis or in the responses to T6P. Thus, screening for suppressors of growth inhibition on 100 mM trehalose will not only uncover mutants in trehalose metabolism or the control thereof but also mutants that overcome T6P mediated changes in carbon allocation. Characterization of the physiological effects of 100 mM trehalose on Arabidopsis seedlings presented in this paper will help to interpret the mutants obtained from the proposed suppressor screen. Mutants capable of growth on 100 mM trehalose have been obtained which are being used to further extend our understanding of how T6P accumulation arrests the seedling growth.

Aknowledgment

We are greatly thankful to Dr. Johannes Hanson for excellent assistance in Q-PCR experiment and Frits Kindt and Ronald Letio for help with confocal microscope and photographs.

References

1- Blazaquez M. A., Santos E., Flores C. L., Martinez-Zapater J. M., Salinas J. and Gancedo C. (1998) Isolation and molecular characterization of the Arabidopsis TPS1 gene, encoding trehalose-6-phosphate synthase. Plant Journal 13: 685-689.

2- Brumfiel, G. (2004) Cell Biology: Just add water. Nature 428:14-15.

3- Crowe J. H., Carpenter J. F. and Crowe L. M. (1998) The role of verification in anhydrobiosis. Annual Review of Physiology 60:73-103.

4- Eastmonad P. J., Van Dijken A. J., Spielman M., Kerr A., Tissier A. F., Dickinson H. G., Jones J. D., Smeekens S. C. and Graham I. A. (2002) Trehalose-6-phosphate synthase 1, which cataltses the first step in trehalose synthesis, is essential for Arabidopsis embryo maturation. Plant Journal 29:225-235.

5- Elbein A. D., PanY. T., Pastuzak I. and Carroll D. (2003) New insights on trehalose: a multifunctional molecule. Glycobiology 13: 17R-27R.

6- Fernandez O., Bethencourt L., Quero A., Sangwan R. S. and Clement C. (2010) Trehalose and plant stress responses: friends or foe? Trends in Plant Science 15: 409-417.

7- Fritzius T., Aechbacher R., Wiemken A. and Wingler A. (2001) Identification of ApL3 expression by trehalose complements the starch- deficient Arabidopsis mutant adg2-1 lacking ApL1, the large subunit of ADP-glucose pyrophosphorylase. Plant physiology 126: 883-889.

8- Garg A. K., Kim J. K., Owens T. G., Ranwala A. P., Choi Y. D., Kochian, L. V. and Wu R. J. (2002) Trehalose accumulation in rice plants confer high tolerance levels to different abiotic stresses. Proceedings of the National Academy of Sciences 99:15898-15903.

9- Holmstrom K. O., Mantyla E., Welin B., Mandal A. and Palva E. T. (1996) Drought tolerance in tobacco. Nature 379:683-684.

10- Iturriaga G., Suarez R. and Nova-Franco B. (2009) Trehalose metabolism: from osmoprotection to siganlling. International Journal of Molecular Science 10: 3793-3819

11- Jang, I. C. Oh S. J. Seo J. S., Choi W. B., Song, S. I., Kim C. H., Kim Y. S., Seo H. S., Choi Y. D., Nahm B. H. and Kim J. K. (2003) Expression of a bifunctional fusion of the E.coli genes for trehalose-6-phosphate phosphatase in transgenic rice plants increase trehalose accumulation and abiotic stress tolerance without stunting growth. Plant physiology 131: 516-524.

12- Jeffery S. and Humphrey G. F. (1975) New spectrophotometric aquations determining chlorophyll a, b, c1 and c2 in higher plants, algae and phytoplankton. Plant Physiology 167:191-194.

13- Leyman B., Van Dijck P. and Thevelein J. M. (2001) An unexpected plethora of trehalose biosynthesis genes in Arabidopsis thaliana. Trends in Plant Science 6:510-513.

14- Mita S., Murano N. and Nakamura K. (1997) Mutants of Arabidopsis thaliana with pleiotropic effects on the expression of the gene for beta-amylase and on the accumulation of anthocyanin that is inducible by sugars. Plant Journal 11:841-851.

15- Muller J., Boller T. and Wiemken A. (1995) Trehalose and trehalase in plants: recent developments. Plant Science 112: 28-35.

16-Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiology 15:473-479.

17- Paul M., Lucia F., Primavesi F., Deveraj J. and Zhang Y. (2008) Trehalose metabolism and signaling. Annual Review of Plant Biology 59: 417-441

18- Pellny T. K., Ghannoum O., Conroy J. P., Schluepmann H., Smeekens S., Andralojc J., Krause, K. P., Goddijn O. and Paul M. J. (2004) Genetic Modification of photosynthesis with E.coli genes for trehalose synthesis. Plant Biotechnology Journal 2: 71-82.

19- Pramanic M. H. and Imai, R. (2005) Functional identification of a trehalose-6-phosphate phosphatase gene that is involved in transient induction of trehalose biosynthesis during chililing stress in rice. Plant Molecular Biology 58:751-762.

20- Ramon M., Rolland F., Thevelein J. M., Van Dijck P. and Leyman B. (2007) ABI4 mediates the effects of exogenous trehalose on Arabidopsis growth and starch breakdown. Plant Mol. Biol., 63: 195-206 .

21- Reignault P., Cogan A., Mucheembled J., Sahraoui A.L.H., Durand, R. and Sancholle M. (2001) Trehalose induces resistance to powedery mildew in wheat. New Phytologist 149: 519-529.

22- Rolland F., Baena-Gonzales E. and Sheen J. (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. Annual Review of Plant Biology 57:675-709.

23- Romero C., Belle J.M., Vaya J. L., Serrano R. and CulianezMacia F. A. (1997) Expression of yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. Planta Journal 201:293-297.

24- Schluepmann H., Pellny T., Van Dijken A., Smeekens S. and Paul M. (2003) Trehalose-6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. Proceedings of the National Academy of Sciences 100: 6849-6854.

25- Schluepmann H., Van Dijken A., Aghdasi M., Wobbes B., Paul M. and Smeekens S. (2004) Trehalose mediated growth inhibition of Arabidopsis seedlings is due to trehalose-6-phosphate accumulation Plant Physiology 135: 879-890.

26- Schluepmann H. and Paul M. J. (2009) Trehalose Metabolites in Arabidopsis—elusive, active and central. The Arabidopsis Book. Rockville, MD: American Society of Plant Biologists.

27- Shima S. Matsui H. Tahara S. and Imai R. (2007) Biochemical characterization of rice trehalose-6-phosphate phosphatases supports distinctive functions of these plant enzymes. FEBS Journal 274:1192.

28- Thevelein J. M. (1984) Regulation of trehalose mobilization in fungi. Microbiology Review 48:42-59.

29- Vandesteene L., Ramon M., Le Roy K., Van Dijck P. and Rolland F. (2010) A single active Trehalose-6-p synthase (TPS) and a family of putative regulatory TPS-like proteins in Arabidopsis. Molecular Plant advance journal 2: 1-14

30- Vogel G. Aeshbacher R. A. Muller J., Boller T. and Wiemken A. (1998) Trehalose-6-phosphate phosphatase from Arabidopsis thailana: identification by functional complementation of the yeast *tps2* mutant. Plant Journal 13:673-683.

31- Wingler A., Fritzius T., Wiekman A., Boller T. and Aeschbacher R. A. (2000) Trehalose induces the ADP-glucose pyophosphorylase gene ApL3, and starch synthesis in Arabidopsis. Planr Physiology 124:105-114.

32- Wolkers W. F., Looper S. A., Fontanilla R. A., Tsvetkova N. M., Tablin F. and Crowe J. H. (2003) Temprature dependence of fluid phase endocytosis coincides with membrane properties of pig plates. Biochim. Biochimica et Biophysica Acta 12:154-163.

33- Zentella R., Mascorro-Gallardo J. O., Van Dijck P., Folch-Mallol J., Bonini B., Van Vaeck C., Gaxiola R., Covarrubiaa A. A., Nieto-Sotelo J., Thevelein J. M. and Iturriga G. (1999) A Selaginella Lepidophylla trehalose-6-phosphate synthase complements growth and stress defects in yeast tps1 mutant. Plant Physiology 119:1473-1482.